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BEITRÄGE ZUR KENNTNIS DES SOG. GEBUNDENEN BLUTZUCKERS.

I. Mitteilung.*

Über das Verhalten des sog. gebundenen Blutzuckers bei der Eiweiss-Fettdiät mit besonderer Berücksichtigung seiner Beziehung zu dem freien Blutzucker.

Von

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(Eingegangen am 16. Dezember 1935)

Wenn man aus frischem Blut die eiweisshaltigen Bestandteile in irgendeiner Weise entfernt, so bekommt man eine wasserklare Flüssigkeit, die bekanntlich die Fähigkeit hat, eine alkalische Lösung von Kupfersalz zu reduzieren. Die in dieser Fraktion enthaltenen reduzierten Substanzen nennt man mit Recht einfach "freier Blutzucker" im etwa dem "gebundenen Blutzucker" entgegengesetzten Sinne des Wortes, worauf ich gleich unten zurückkommen werde. Hydrolysiert man dasselbe Blut vor der Entfernung der Eiweisssubstanzen mit einer nicht zu konzentrierten Mineralsäure, so wird das nachher gefundene reduzierende Vermögen im allgemeinen mehr oder weniger grösser. Seit dieser fundamentalen Entdeckung Figuiers (1855) liegen zahlreiche Untersuchungen darüber vor, die sich mit der Natur derselben Substanzen beschäftigen, ohne aber miteinander übereinzustimmen.

Das Reduktionsvermögen des nichthydrolysierten Blutes d.h. das des sog. freien Blutzuckers ist zum weitaus grössten Teile auf Rechnung einer Zuckerart zu schreiben, die mit der gewöhnlichen Glucose identisch oder wenigstens ihr nahe verwand ist. Die durch

* Die vorliegende Mitteilung wurde am 4. März 1935 in der Gesellschaft für Gastroenterologie in Kyoto öffentlich vorgetragen.

Hydrolyse zugenommenen reduzierenden Substanzen hatten Bierry und Randoin-Fandard (1914) als Sucre protéidique, und Condorelli (1924) als Zucchero combinato bezeichnet, weil diese Autoren sie für an das Bluteiweiss gebundene Glucose hielten. Diese Meinung teilen auch Lépine, Pavy, Boulud, Langstein, Frank, Best, Abderhalden, Quariariello, Iizuka, Katayama, u.a..

Bigwood und Wuillot (1928), Somogyi und Iketani (1927) sind aber der Auffassung, dass diese Substanzen nicht schlechthin als Zucker bezeichnet werden können, weil sie nur wenig oder gar nicht zu vergären seien. Vor kurzem kamen Tateishi (1932) und Morimune (1934) zur Meinung, dass diese Substanzen mit dem Eiweisskörper fest verbunden und zum Teil (30–40%) vergärbar seien. Andererseits kamen Nakamura (1929) und Furuyama (1933) auf Grund ihrer Untersuchungen zu dem Schluss, dass es sich bei der Zunahme des Reduktionsvermögens nach Hydrolyse des Blutes wesentlich um die Abbauprodukte des Bluteiweisses wie z.B. Glukosamin und zum kleinen Teil auch um die Glucose aus Glykogen sowie das Kreatinin aus Kreatin handelt; sie bezeichneten daher diese Substanzen statt der Benennung "gebundener Zucker" als "gebundene reduzierende Substanzen". Jedenfalls sind sich die verschiedenen Autoren über die Begriffe der wahren Natur dieser Substanzen nicht einig. Im Folgenden bezeichne ich die betreffenden Substanzen der Bequemlichkeit halber vorläufig als "sog. gebundenen Blutzucker".

Was die Bedeutung des sog. gebundenen Zuckers sowohl in der Physiologie, als auch in der Pathologie anbetrifft, so gibt es auch zahlreiche Veröffentlichungen, die aber zu keinem befriedigenden Schluss gelangen. Allerdings glauben die meisten Autoren, dass der gebundene Zucker eine nicht zu unterschätzende Rolle im Kohlenhydratstoffwechsel spiele.

Nach dem Ort, wo der sog. geb. Zucker etwa gespalten bzw. gebildet wird, ist auch von mehreren Forschern gesucht worden. Eine Reihe von Autoren halten Leber, Lunge sowie Niere für Organe, die eine Fähigkeit haben, den sog. geb. Zucker zu spalten, weil sie in dem venösen Blut, das durch diese Organe strömt,

mehr freien Zucker und weniger sog. geb. Zucker, als in dem arteriellen gefunden haben. Andererseits haben Tateishi (1930), Imanishi (1930) und Takuwa (1931) festgestellt, dass bei Funktionsstörungen dieser Organe der Gehalt des Blutes an sog. geb. Zucker grösser als sonst gefunden wird. Sehr wenig erforscht ist es heute noch, an welchem Ort der sog geb. Zucker gebildet, d.h. aus seinen Bestandteilen in die gekuppelte Form synthetisiert wird. Bierry u.a. haben die Angabe gemacht, dass die Muskulatur beim Aufbau des sog. geb. Zuckers eine bedeutende Rolle spielen soll. Auf Grund zahlreicher Untersuchungen sind Iizuka, einer der hervorragendesten japanischen Forscher auf diesem Gebiete, und seine Schüler der Ansicht, dass das retikuloendotheriale System im weitgehendem Masse an seiner Bildung beteiligt ist. Alles in allem bedarf der sog. geb. Zucker noch weiterer Erforschung.

Andererseits haben Kageura (1922) und Staub (1922), unabhängig von einander, festgestellt, dass die kohlenhydratarme Diät, welche wesentlich aus Eiweiss und Fett besteht, die Assimilationsfähigkeit für Zucker beim Menschen und Hunde deutlich herabsetzt, und ferner, dass die geschädigte Zuckerassimilation sich durch Kohlenhydratzusatz zur kohlenhydratarmen Grundkost auffallend bessert. Durch seine tierexperimentellen Untersuchungen kam schliesslich Kageura zu der Meinung, dass für die durch die genannte Ernährungsweise verursachte Beeinträchtigung der Zuckerassimilationskraft die Herabsetzung der Glykogenbildung in der Leber sicher ein ätiologisches Moment darstellt. Bei solcher Sachlage sei es von Bedeutung, die Beeinflussung des sog. geb. Blutzuckers durch Eiweiss-Fettdiät; die die Fähigkeit, Zucker zu assimilieren, deutlich schädigt, und die Beziehung, die zwischen dem freien und sog. geb. Blutzucker besteht, zu studieren.

Als Versuchstiere wurden fünf gesunde ausgewachsene Hunde verwendet, weil nach den Erfahrungen Kageura's dieses Tier für Untersuchungen über den Kohlenhydratstoffwechsel sehr geeignet ist. Die Hunde wurden eine bestimmte Zahl von Tagen zuerst, wie bei uns üblich, mit kohlenhydratreicher gemischter Kost, dann mit Eiweiss-Fettdiät, die aus magerem Rindfleisch und

Schweineschmalz besteht, genährt, bekamen dann bei Gleichhalten der Eiweiss-Fettration eine gewisse Menge Kohlenhydrat, gekochten Reis, dem Fütter zugesetzt. Dabei wurde der Kaloriengehalt der Nahrung, meinen früheren Erfahrungen entsprechend, so bestimmt, dass das Körpergewicht der Tiere innerhalb der Beobachtungszeit keine bedeutende Schwankung erfuhr. In der Ernährungsperiode, die je nach dem Fall 2–4 Tage lang dauerte, wurde den Tieren nach 24-stündigem Fasten eine bestimmte Menge (für denselben Hund immer gleich, aber nicht bei allen Tieren dieselbe) Traubenzucker in 200 ccm warmem Wasser gelöst durch Sonde in den Magen eingeführt und danach der Gehalt des freien und des sog. geb. Blutzuckers gleichzeitig stündlich 3 Stunden lang vergleichend untersucht. Zu diesem Zweck wurde 3 ccm Blut an der Ohrvene dem Tier entnommen, um davon eine gewisse Menge Fluoridplasma zu gewinnen. Zur Bestimmung des sog. geb. Zuckers dieses Plasmas verwendete ich die Mikrobestimmungsmethode Iizukas (1928) und zu der des freien Zuckers desselben die Methode Hagedorn-Jensens (1923). Bei der Bestimmung des sog. geb. Zuckers wurde der Wert desselben nach der Rechnungsmethode Nakamuras (1934) (freier Blutzuckergehalt $\times$ 0,082) addiert, weil die Glucose durch Behandlung mit der Bestimmungsmethode des sog. geb. Zuckers an ihrem reduzierenden Vermögen um 8,2% verliert. In jeder Periode wurde genau dieselbe Untersuchung sowohl über den freien, als auch den sog. geb. Zucker stets zwei Mal ausgeführt, um die Mittelwerte zwischen den gefundenen Zahlen zu bekommen. Die Versuchsresultate an fünf Hunden sind übersichtlich in einer Tabelle dargestellt. Zur leichteren Orientierung ist ausserdem noch eine Abbildung beigefügt, die die an dem Hund Nr. 3 gewonnenen Ergebnisse betrifft.

Wie aus der Tabelle ersichtlich ist, stimmen die Resultate meiner Untersuchungen an fünf Hunden im grossen und ganzen miteinander ganz gut überein. Die Nüchternwerte des freien sowie des sog. geb. Blutzuckers werden durch die oben genannten verschiedenen Ernährungsweisen gar nicht eindeutig beeinflusst. Bei Eiweiss-Fettdiät kommt aber die alimentäre Steigerung des freien Blutzuckers nach Zuckerdarreichung auffallend stärker und länger

TABELLE.

Nr. 1. 40 g Traubenzucker dargereicht.

Diät (pro Tag) Untersuchungsperiode (Rindfleisch / gekocht. Reis / Schweineschmalz)	Datum	Körpergewicht	Blutzuckergehalt (%) fr. Z. od. geb. Z.	vor der Zuckerzufuhr	1St. n.d. Zuckerzufuhr	2St.	3St.
	17/XII.	kg.	fr. Z.	0,079	0,119	0,069	0,081
		9,3	geb. Z.	0,104	0,099	0,114	0,112
Gewöhnliche Kost	19/ „	9,3	fr. Z.	0,081	0,115	0,071	0,078
(70 g) (240 g) (18 g)			geb. Z.	0,103	0,105	0,095	0,099
19/XII.——25/XII.	22/ „	9,3	fr. Z.	0,082	0,168	0,141	0,075
			geb. Z.	0,093	0,065	0,064	0,095
Eiweiss-Fettdiät	26/ „	9,6	fr. Z.	0,076	0,176	0,131	0,081
(210 g) (42 g)			geb. Z.	0,099	0,059	0,086	0,082
26/IIX.——29/XII.	28/ „	9,4	fr. Z.	0,082	0,134	0,081	0,082
			geb. Z.	0,104	0,107	0,096	0,104
Kohlenhydratzusatz	30/ „	9,8	fr. Z.	0,090	0,122	0,066	0,093
(210 g) (100 g) (42 g)			geb. Z.	0,099	0,097	0,102	0,083

Nr. 2. 50 g Traubenzucker dargereicht.

Diät (pro Tag) Untersuchungsperiode (Rindfleisch / gekocht. Reis / Schweineschmalz)	Datum	Körpergewicht	Blutzuckergehalt (%) fr. Z. od. geb. Z.	vor der Zuckerzufuhr	1St. n.d. Zuckerzufuhr	2St.	3St.
	21/XII.	kg.	fr. Z.	0,073	0,113	0,090	0,080
		14,3	geb. Z.	0,088	0,094	0,087	0,087
Gewöhnliche Kost	26/ „	14,2	fr. Z.	0,067	0,101	0,094	0,081
(110 g) (380 g) (28 g)			geb. Z.	0,089	0,093	0,070	0,083
26/XII.——29/XII.	28/ „	14,0	fr. Z.	0,068	0,180	0,150	0,070
			geb. Z.	0,109	0,078	0,082	0,116
Eiweiss-Fettdiät	30/ „	14,1	fr. Z.	0,079	0,190	0,185	0,079
(300 g) (70 g)			geb. Z.	0,094	0,066	0,065	0,088
30/XII.—— 6/I.	4/ I	14,4	fr. Z.	0,079	0,161	0,123	0,079
			geb. Z.	0,098	0,069	0,093	0,081
Kohlenhydratzusatz	7/ „	14,3	fr. Z.	0,081	0,168	0,100	0,068
(300 g) (100 g) (70 g)			geb. Z.	0,119	0,107	0,094	0,095

Nr. 3. 40 g Traubenzucker dargereicht.

Diät (pro Tag)	Datum	Körpergewicht	Blutzuckergehalt (%)				
Untersuchungsperiode (Rindfleisch) (gekocht. Reis) (Schweineschmalz)			fr. Z. od. geb. Z.	vor der Zuckerzufuhr	1St. n.d. Zuckerzufuhr	2St.	3St.
	16/ I.	kg.	fr. Z.	0,100	0,131	0,117	0,073
		10,5	geb. Z.	0,100	0,096	0,090	0,105
Gewöhnliche Kost							
(80 g) (270 g) (21 g)	18/ „	10,5	fr. Z.	0,087	0,131	0,114	0,089
			geb. Z.	0,105	0,089	0,100	0,105
18/I.———23/I.	21/ „	10,4	fr. Z.	0,092	0,206	0,119	0,092
			geb. Z.	0,086	0,063	0,082	0,087
Eiweiss-Fettdiät							
(240 g) (45 g)	24/ „	10,2	fr. Z.	0,090	0,183	0,165	0,090
			geb. Z.	0,083	0,051	0,080	0,081
24/I.———27/I.	26/ „	10,3	fr. Z.	0,092	0,122	0,117	0,081
			geb. Z.	0,074	0,072	0,079	0,074
Kohlenhydratzusatz							
(240 g) (100 g) (45 g)	28/ „	9,9	fr. Z.	0,091	0,130	0,128	0,082
			geb. Z.	0,085	0,079	0,083	0,089

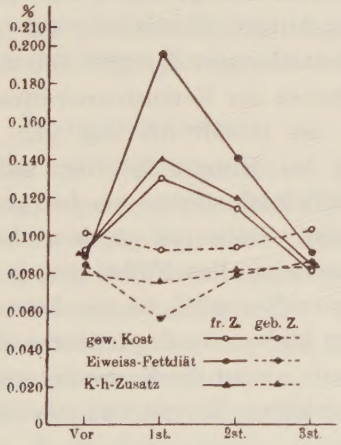
Nr. 4. 40 g Traubenzucker dargereicht.

Diät (pro Tag)	Datum	Körpergewicht	Blutzuckergehalt (%)				
Untersuchungsperiode (Rindfleisch) (gekocht. Reis) (Schweineschmalz)			fr. Z. od. geb. Z.	vor der Zuckerzufuhr	1St. n.d. Zuckerzufuhr	2St.	3St.
	18/ I.	kg.	fr. Z.	0,089	0,112	0,091	0,093
		8,6	geb. Z.	0,078	0,083	0,081	0,087
Gewöhnliche Kost							
(60 g) (210 g) (16 g)	21/ „	8,5	fr. Z.	0,092	0,124	0,126	0,097
			geb. Z.	0,092	0,085	0,083	0,078
21/I.———27/I.	24/ „	8,3	fr. Z.	0,094	0,231	0,219	0,201
			geb. Z.	0,082	0,067	0,068	0,068
Eiweiss-Fettdiät							
(210 g) (40 g)	28/ „	8,5	fr. Z.	0,093	0,276	0,260	0,162
			geb. Z.	0,101	0,072	0,068	0,079
28/I.——— 3/II.	31/ „	8,4	fr. Z.	0,106	0,161	0,134	0,081
			geb. Z.	0,107	0,104	0,100	0,096
Kohlenhydratzusatz							
(210 g) (100 g) (40 g)	3/II.	8,6	fr. Z.	0,095	0,172	0,136	0,087
			geb. Z.	0,105	0,105	0,100	0,105

Nr. 5. 40 g Traubenzucker dargereicht.

Diät (pro Tag)	Datum	Körpergewicht	Blutzuckergehalt (%)				
Untersuchungsperiode (Rindfleisch) (gekocht. Reis) (Schweineschmalz)			fr. Z. od. geb. Z.	vor der Zuckerzufuhr	1St. n. d. Zuckerzufuhr	2St.	3St.
Gewöhnliche Kost (75 g) (220 g) (16 g)	6/II.	kg. 8,3	fr. Z.	0,072	0,112	0,110	0,085
			geb. Z.	0,096	0,089	0,086	0,091
	9/„	8,4	fr. Z.	0,076	0,103	0,092	0,067
			geb. Z.	0,107	0,087	0,081	0,082
10/II.———15/II. Eiweiss-Fettdiät (200 g) (40 g)	13/„	8,4	fr. Z.	0,089	0,190	0,065	0,068
			geb. Z.	0,085	0,054	0,078	0,079
	16/„	8,5	fr. Z.	0,081	0,177	0,081	0,063
			geb. Z.	0,093	0,065	0,082	0,091
16/II.———21/II. Kohlenhydratzusatz (200 g) (100 g) (40 g)	19/„	8,3	fr. Z.	0,089	0,138	0,129	0,069
			geb. Z.	0,110	0,093	0,096	0,106
	22/„	8,4	fr. Z.	0,092	0,150	0,127	0,074
			geb. Z.	0,106	0,105	0,106	0,106

Abb. Hund Nr. 3.



andauernd, als bei gemischter kohlenhydratreicher Kost zur Erscheinung und dieser abnorm gesteigerte hyperglykämische Wert tritt in der nachfolgenden Periode von Kohlenhydratzusatz deutlich schwächer auf als zuvor, sodass er dabei sich wieder dem bei

gemischter Nahrung nähert. Mit der oben erwähnten Beobachtung Kageuras, dass Eiweiss-Fettdiät die zuckerassimilierende Fähigkeit des Organismus deutlich herabsetzt und diese geschädigte Funktion durch Kohlenhydratzusatz zur Kost leicht wieder gebessert wird, stimmen meine Versuchsresultate vollkommen überein. Der Gehalt des Blutes an sog. geb. Zucker nach Zuckerzufuhr zeigt bei gemischter Nahrung keine erhebliche Veränderung, doch ist dabei eine geringfügige Herabsetzung desselben nicht ganz zu verkennen und zwar neigt der einnmal verminderte Wert dazu etwa drei Stunden nach Zuckerzufuhr wieder zu steigen. Die Resultate stimmen wesentlich mit den Angaben von mehreren Autoren (Condorelli(1924), Brugi(1925), Toscano(1928), Imanishi (1929), Hukui u. Nakamura (1933) überein. Condorelli (1924) gab an, dass nach Verarbreichung von 20 g Glucose an ein gesundes Individuum die Hy-S-Kurve (d.h. Kurve der gesamten Substanzen, die nach Hydrolyse mit Säure stärker reduzierend wirken als zuvor) des Plasmas zuerst absinkt, bisweilen sogar auf Null, dann wiederum ansteigt und schlieslich wieder normal wird. Meine diesbezügliche Kurve zeigt eine Form, die der Hauptsache nach mit der dieses Autors gleichdeutig ist, wenngleich sie im einzelnen geringe Abweichungen darstellt, die m.E. wesentlich auf kleinen Verschiedenheiten der Versuchsanordnung beruhen. Ganz anders verhält sich der Gehalt des sog. geb. Blutzuckers nach Zuckerverabreichung bei Eiweiss-Fettdiät, sodass hier die alimentäre Senkung auffallend stärker als bei gemischter Nahrung zum Ausdruck kommt. Dabei ist es bemerkenswert, dass der tiefste Punkt der Kurve in allen Fällen gerade eine Stunde nach Zuckerdarreichung getroffen wird, wo die Kurve des freien Blutzuckers ein Fastigium bildet. In der letzteren Ernährungsperiode von Kohlenhydratzusatz nimmt die Kurve des sog. geb. Blutzuckers wieder ein deutlich erhöhtes Niveau ein, das dem bei gemischter Kost sehr nahe liegt.

Der Einfluss der Ernährung auf den Gehalt des sog. Blutzuckers ist bisher überhaupt sehr wenig untersucht worden. Es ist eine neu gefundene Tatsache, dass der sog. geb. Blutzuckergehalt nach peroraler Zuckerzufuhr durch vorangehende Eiweiss-

Fettdiät deutlich herabgesetzt wird und diese Senkung des sog. geb. Zuckers durch Kohlenhydratzusatz zur Grundkost auffallend paralysiert wird. Es ist von grossem Interesse, dass Kohlenhydrat in der Nahrung, das auf die durch Eiweiss-Fettdiät erhöhte Steigerung des freien Blutzuckers nach Zuckerherabreichung deutlich dämpfend einwirkt, auch auf die durch die genannte Diät verstärkte alimentäre Senkung des sog. geb. Blutzuckers einen paralysierenden Einfluss ausübt. Mit anderen Worten gilt das von Kageura in betreff des freien Blutzuckers gefundene Gesetz, dass Kohlenhydrat in der Nahrung einerseits und Eiweiss-Fett in derselben andererseits den Kohlenhydratstoffwechsel im entgegengesetzten Sinne (d.h. antagonistisch) beeinflussen, auch von dem sog. geb. Blutzucker. Ausserdem ist es merkwürdig, dass der Kurvengipfel des freien Blutzuckers zeitlich gerade mit dem Tal der Kurve des sog. geb. Blutzuckers zusammenfällt. Die Frage, wie die genannte Wirkung der Eiweiss-Fettdiät auf den Spiegel des sog. geb. Blutzuckers nach Zuckerzufuhr sich äussert, bleibt noch offen. Ferner bedürfen das Verhältnis, das zwischen dem freien und sog. geb. Blutzucker besteht, und die Rolle der verschiedenen Organe bzw. Systeme bei der Regulierung des Gehalts des sog. geb. Blutzuckers noch näherer Untersuchung.

Zusammenfassung.

1. Der Gehalt des sog. geb. Blutzuckers wird durch vorangehende Eiweiss-Fettdiät gar nicht deutlich beeinflusst.

2. Bei gemischter kohlenhydratreicher Nahrung wird der Gehalt des Blutes an sog. geb. Zucker durch perorale Zuckerzufuhr nur in unbedeutendem Ausmasse herabgesetzt.

3. Diese alimentäre Senkung des sog. geb. Blutzuckers wird durch Eiweiss-Fettdiät erheblich verstärkt.

4. Diese durch Eiweiss-Fettdiät verstärkte Senkung des sog. geb. Blutzuckers nach Zuckerdarreichung wird durch Kohlenhydratzusatz zur Kost paralysiert.

5. Der höchste Punkt der Kurve des freien Blutzuckers fällt zeitlich mit dem tiefsten Punkt der Kurve des sog. geb. Blutzuckers zusammen.

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II. Mitteilung.

Über das Verhalten des sog. gebundenen Blutzuckers bei Hunger mit besonderer Berücksichtigung seiner Beziehung zu dem freien Blutzucker.

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Lehman (1874) und Claude Bernard (1877) wiesen schon lang darauf hin, dass die kohlenhydratassimilierende Fähigkeit der Organismen (auf Grund von Untersuchungen des freien Blutzuckers) durch Hunger herabgesetzt wird, sodass nach einer Zuckerzufuhr hier eine Glykosurie viel leichter zum Vorschein kommt. Seitdem wurde diese sogenannte "Hungerdiabetes" (Hofmeisters, 1890) von zahlreichen Autoren wie Barrenscheen (1914), Lindberg (1918), Staub (1922), Traugott (1923), Nagasue (1925), Schiff und Mitarbeitern (1930), Ikejiri (1933) u.s.w. erschöpfend untersucht. Vor Kurzem hat Ikejiri (1933) festgestellt, dass die durch Hunger geschädigte zuckerassimilierende Kraft durch Wiederverabreichung von kohlenhydratreicher gemischter Nahrung in relativ kurzer Zeit wieder gebessert wird.

Andererseits haben Kageura (1922) und Staub (1922), unabhängig voneinander, gefunden, dass Eiweiss-Fettnahrung die Assimilationsfähigkeit der Organismen für Zucker deutlich herabsetzt, und ferner Kageura, dass diese geschädigte Assimilationskraft durch Kohlenhydratzusatz zur Grundkost leicht wieder gebessert wird. Nun unterliegt es keinem Zweifel mehr, dass der absolute Hunger wie auch die Ernährung mit kohlenhydratarmer

Kost auf den Kohlenhydratstoffwechsel einen schädigenden Einfluss ausüben und ferner, dass jede geschädigte Assimilationskraft für Zucker durch einige Tage andauernde kohlenhydratreiche Nahrung wiederhergestellt wird.

Bei Hunger liegen auch zahlreiche Untersuchungen über den sog. gebundenen Blutzucker vor, ohne aber miteinander übereinzustimmen. Kombinierten Lépine und Boulud (1910) Blutverlust mit Hungern, so sank der sog. geb. Blutzucker gleichfalls ab; Glykogen und Fett waren zum grössten Teil verschwunden, der freie Blutzucker hingegen stieg an. Auf Grund ihrer Untersuchungen sind Condorelli (1924), Grevenstuk (1929), Caltaviano (1929) der Ansicht, dass 24-stündiger Hunger bei Kaninchen schon den sog. geb. Blutzucker beträchtlich herabsetzen kann. Scott und Best (1924) beobachteten bei hungernden Kaninchen und Hunden durch saure Hydrolyse eine nur geringe Zunahme des reduzierenden Vermögens des Blutes.

Wenn man solche Extreme wählt, dass die Grenzen des Physiologischen weit überschritten werden—lässt man z.B. Hunde tothungern—, so steigt der sog. geb. Blutzucker mit der Zeit allmählich zu höheren Werten an, sodass in der Agonie, wo freier Blutzucker und Organglykogen auf ihr Minimum angelangt sind, der sog. geb. Blutzucker gerade maximal ist (Lépine, 1921; Bierry und Fandard, 1913). In seiner Veröffentlichung über das Hungern von langer Dauer hat Okamoto (1931) berichtet, dass der Wert des sog. geb. Blutzuckers bei Kaninchen in der überwiegenden Mehrzahl der Fälle vom Anfangsstadium des Hungerns an bis zum Spätstadium auffallend zunimmt, bei den übrigen Fällen aber nicht, und der des freien Blutzuckers anfangs absinkt oder wenigstens zum Abzusinken neigt und im Spätstadium dagegen ansteigt.

In meiner vorangehenden Arbeit habe ich mitgeteilt, dass beim Hunde die nach Zuckerzufuhr aufgetretene Senkung des sog. geb. Blutzuckers und die alimentäre Steigerung des freien Blutzuckers bei Ernährung mit kohlenhydratarmer Eiweiss-Fettkost viel deutlicher auftritt als bei normaler Ernährung, und ferner, dass sowohl die durch Eiweiss-Fettdiät verursachte Schädigung der Zucker-

assimilationsfähigkeit als auch die verstärkte alimentäre Senkung des sog. geb. Blutzuckers durch Kohlenhydratzusatz zur Grundkost auffallend paralysiert wird. Die noch offen bleibende Frage, wie der Hunger von langer Dauer den sog. gab. Blutzucker nach Zuckerzufuhr beeinflusst und ferner ob die dadurch herbeigeführte alimentäre Veränderung des sog. geb. Blutzuckers durch Wiederverabreichung von kohlenhydratreicher Nahrung beseitigt wird, experimentell zu erledigen, ist Aufgabe dieser Untersuchung.

Als Versuchstiere wurden in allen Fällen männliche ausgewachsene Hunde benützt, die schon lang mit kohlenhydratreicher gemischter Kost ernährt worden waren. Die Hunde wurden zuerst eine bestimmte Zahl von Tagen, wie es bei uns üblich ist, weiter mit kohlenhydratreicher Kost genährt und dann dem Fasten von ein- bzw. dreiwöchiger Dauer unterzogen. Jedoch wurde den Hunden auch während der Hungerperiode nur Wasser hinreichend gegeben, um einerseits den frühzeitigen Tod durch Wasserverlust zu vermeiden und weil andererseits die Blutentnahme aus den Ohrvenen bei der Untersuchung nach meiner Erfahrung sehr leicht auszuführen ist. Dann wurde die alimentäre Veränderung des freien sowie des sog. geb. Blutzuckers in jeder oben erwähnten Periode vergleichend betrachtet. Die meisten diesbezüglichen Prozeduren wurden in derselben Weise ausgeführt, wie sie in meiner vorangehenden Mitteilung beschrieben sind, jedoch wurde einigen Hunden in der Versuchsreihe von langdauerndem Hunger unter Berücksichtigung der Körpergewichtsabnahme eine etwas geringere Menge Traubenzucker als bei gemischer Nahrung gegeben.

Die Versuchsresultate an elf Hunden seien hier übersichtlich in Tabellenform mitgeteilt.

Wie die Tabelle zeigt, wird die Assimilationsfähigkeit für Zucker durch Hunger bedeutend herabgesetzt, d.h. die Hyperglykämie nach Zuckerzufuhr tritt hier weit höher und länger dauernd in Erscheinung als bei kohlenhydratreicher gemischter Nahrung, was mit der Angabe Ikejiris (1933) vollkommen übereinstimmt, wobei der Wert des sog. geb. Blutzuckers mit der Steigerung des freien Blutzuckers zusammen ansteigt. Dabei zeigt der Nüchternwert des freien Blutzuckers nach der ein- bzw.

TABELLE I. Einwöchiger Hunger.

Nr. d. Hundes	Untersuchungsperiode	Datum	Körpergewicht	Menge d. gegebenen Zuckers	Blutzuckergehalt (%)				
					fr. Z. od. geb. Z.	vor der Zuckerzufuhr	1St. n. d. Zuckerzufuhr	2St.	3St.
Nr. 6	gewöhnliche Kost	16/IV	kg. 11,2	g. 40	fr. Z.	0,087	0,112	0,089	0,090
					geb. Z.	0,117	0,117	0,121	0,116
	seit 17/VI Hunger	23/ „	9,1	40	fr. Z.	0,091	0,275	0,267	0,149
					geb. Z.	0,107	0,134	0,142	0,128
Nr. 7	gewöhnliche Kost	16/ „	11,0	40	fr. Z.	0,083	0,126	0,085	0,089
					geb. Z.	0,120	0,115	0,112	0,123
	seit 17/VI Hunger	23/ „	9,4	40	fr. Z.	0,086	0,202	0,193	0,084
					geb. Z.	0,115	0,118	0,103	0,099
Nr. 8	gewöhnliche Kost	19/ „	8,5	35	fr. Z.	0,100	0,117	0,110	0,085
					geb. Z.	0,115	0,120	0,123	0,116
	seit 20/VI Hunger	26/ „	6,9	35	fr. Z.	0,096	0,208	0,208	0,199
					geb. Z.	0,117	0,133	0,126	0,139
Nr. 9	gewöhnliche Kost	19/ „	6,3	25	fr. Z.	0,073	0,098	0,064	0,078
					geb. Z.	0,122	0,118	0,119	0,131
	seit 20/VI Hunger	26/ „	5,1	25	fr. Z.	0,065	0,331	0,331	0,315
					geb. Z.	0,138	0,162	0,158	0,139
Nr. 10	gewöhnliche Kost	12/VIII	7,9	40	fr. Z.	0,099	0,125	0,140	0,107
					geb. Z.	0,105	0,105	0,107	0,104
	seit 13/VIII Hunger	20/ „	7,0	40	fr. Z.	0,086	0,220	0,252	0,214
					geb. Z.	0,092	0,104	0,115	0,128
Nr. 11	gewöhnliche Kost	12/ „	9,5	40	fr. Z.	0,078	0,140	0,109	0,104
					geb. Z.	0,106	0,099	0,106	0,109
	seit 13/VIII Hunger	20/ „	8,1	40	fr. Z.	0,084	0,200	0,234	0,186
					geb. Z.	0,102	0,124	0,127	0,123

Dreiwöchiger Hunger.

Nr. d. Hundes	Untersuchungsperiode	Datum	Körpergewicht	Menge d. gegebenen Zuckers	Blutzuckergehalt (%)				
					fr. Z. od. geb. Z.	vor der Zuckerzufuhr	1St. n. d. Zuckerzufuhr	2St.	3St.
Nr. 12	gewöhnliche Kost	7/V	kg. 11,1	g. 40	fr. Z.	0,096	0,115	0,096	0,092
					geb. Z.	0,109	0,120	0,119	0,120
	seit 8/V Hunger	27/ „	7,9	35	fr. Z.	0,088	0,258	0,285	0,297
					geb. Z.	0,077	0,138	0,161	0,130
Nr. 13	gewöhnliche Kost	7/ „	9,8	40	fr. Z.	0,088	0,120	0,079	0,087
					geb. Z.	0,129	0,131	0,123	0,127
	seit 8/V Hunger	27/ „	7,2	35	fr. Z.	0,095	0,300	0,375	0,408
					geb. Z.	0,085	0,178	0,187	0,190
Nr. 14	gewöhnliche Kost	10/ „	7,6	40	fr. Z.	0,097	0,127	0,125	0,104
					geb. Z.	0,090	0,087	0,100	0,090
	seit 11/V Hunger	30/ „	5,5	40	fr. Z.	0,079	0,327	0,387	0,486
					geb. Z.	0,074	0,132	0,148	0,166
Nr. 15	gewöhnliche Kost	10/ „	13,8	50	fr. Z.	0,108	0,129	0,079	0,093
					geb. Z.	0,108	0,115	0,111	0,112
	seit 11/V Hunger	30/ „	10,7	50	fr. Z.	0,097	0,270	0,312	0,162
					geb. Z.	0,064	0,091	0,115	0,130
Nr. 16	gewöhnliche Kost	5/VIII	9,6	40	fr. Z.	0,084	0,103	0,080	0,088
					geb. Z.	0,100	0,093	0,096	0,098
	seit 8/VIII Hunger	27/ „	6,8	35	fr. Z.	0,064	0,238	0,326	0,338
					geb. Z.	0,081	0,105	0,132	0,159

dreiwöchigen Hungerperiode keine nennenswerte Veränderung, und der des sog. geb. Blutzuckers nach der einwöchigen Hungerperiode auch nicht, doch tritt derselben Wert nach der dreiwöchigen Hungerperiode ziemlich niedriger als bei gewöhnlicher Nahrung auf. Ferner kann man bemerken, dass je länger die Dauer des Hungers währt, desto stärker die Steigerung des freien sowie des sog. geb. Blutzuckers nach Zuckerzufuhr zum Vorschein kommt.

In der Versuchsreihe von eine Woche hungernden Hunden befindet sich der Gipfel der Kurve am freien Blutzucker meistens an der 1–2 Stunde nach Zuckerzufuhr und der am sog. geb. Blutzucker mit ihm zugleich oder etwas verzögert, und in der Versuchsreihe von drei Wochen hungernden Hunden wird dagegen der Gipfel des freien sowie des sog. geb. Blutzuckers in der Mehrzahl der Fälle an der 3. Stunde nach Zuckerzufuhr gefunden. Es ist merkwürdig, dass bei Hunger die Kurve des sog. geb. Blutzuckers nach Zuckerzufuhr wider Erwarten ganz anders ist als bei Eiweiss-Fettdiät, während diese bekanntlich auf den Wert des freien Blutzuckers einen mit Hunger gleichartigen Einfluss ausübt; d.h. beim Hunger bewegen sich die Kurven des freien sowie des sog. geb. Blutzuckers nach Zuckerzufuhr in gleichnamiger Richtung, während die Kurven der beiden Substanzen bei Eiweiss-Fettnahrung, wie in meiner vorangehenden Mitteilung schon erwähnt, in entgegengesetzter Richtung voneinander gehen. Mit anderen Worten üben der absolute Hunger wie auch die Ernährung mit Eiweiss-Fett auf den Wert des freien Blutzuckers einen gleichartigen schädigenden Einfluss aus, doch scheinen sie auf den Spiegel des sog. geb. Blutzuckers einen gerade entgegengesetzten Einfluss auszuüben. In der alimentären Steigerung am freien sowie sog. geb. Blutzucker bei langdauerndem Hunger ist kein ausgesprochener Unterschied zwischen den einigen besonderen Fällen (Nr. 12. 13. 16.), bei denen eine etwas geringere Menge Traubenzucker als bei gemischter Nahrung zugeführt wurde, und den übrigen Fällen (Nr. 14. 15.) nachzuweisen.

Die Frage nach dem Einfluss der Wiederaufnahme der Fütterung auf die durch Hunger herbeigeführte Steigerung des sog. geb. Blutzuckers nach Zuckerzufuhr bleibt noch offen. Um diese Frage

Tabelle II.

Nr. d. Hundes	Untersuchungsperiode	Datum	Körpergewicht	Menge d. gegebenen Zuckers	Blutzuckergehalt (%)				
					fr. Z. od. geb. Z.	vor der Zuckerzufuhr	1St. n. d. Zuckerzufuhr	2St.	3St.
Nr. 10	gewöhnliche Kost	12/VIII	kg. 7,9	g. 40	fr. Z.	0,099	0,125	0,140	0,107
					geb. Z.	0,105	0,105	0,107	0,104
	seit 13/VIII Hunger	20/ „	7,0	40	fr. Z.	0,086	0,220	0,252	0,214
					geb. Z.	0,092	0,104	0,115	0,128
	seit 20/VIII gewöhn. Kost	23/ „	7,4	40	fr. Z.	0.083	0,091	0,096	0,100
					geb. Z.	0,101	0,091	0,093	0,121
Nr. 11	gewöhnliche Kost	12/ „	9,6	40	fr. Z.	0,078	0,140	0,109	0,104
					geb. Z.	0,106	0,099	0,106	0,109
	seit 13/VIII Hunger	20/ „	8,1	40	fr. Z.	0,084	0,200	0,234	0,186
					geb. Z.	0,102	0,124	0,127	0,123
	seit 20/VIII gewöhn. Kost	23/ „	8,4	40	fr. Z.	0,094	0,103	0,113	0,103
					geb. Z.	0,103	0,107	0,112	0,108
Nr. 16	gewöhnliche Kost	5/ „	9,6	40	fr. Z.	0,084	0,103	0,080	0,088
					geb. Z.	0,100	0,093	0,096	0,098
	seit 8/VIII Hunger	27/ „	6,8	35	fr. Z.	0,064	0,238	0,326	0,338
					geb. Z.	0 081	0,105	0,132	0,159
	seit 27/VIII gewöhnliche Kost	30/ „	7,1	35	fr. Z.	0,092	0,124	0,115	0,107
					geb. Z.	0,089	0,088	0,101	0,099
		3/ „	7,7	35	fr. Z.	0,110	0,145	0,134	0,096
					geb. Z.	0,103	0,094	0,093	0,106

zu klären, wurden die Hunde (Nr. 10. 11. 16.) nach der Hungerperiode einige Tage lang wieder mit kohlenhydratreicher gemischter Nahrung genährt und die dabei eingetretenen alimentären Veränderungen am freien wie auch sog geb. Blutzucker in jeder Periode vergleichend betrachtet. Die einzelnen Daten sind in der folgenden Tabelle zusammengestellt.

Wie aus der Tabelle ersichtlich ist, wird nicht nur die durch Hunger verursachte verstärkte Steigerung des freien Blutzuckers nach Zuckerzufuhr, sondern auch die dadurch hervorgerufene alimentäre Steigerung des sog. geb. Blutzuckers durch Wiederverabreichung kohlenhydratreicher gemischter Nahrung in relativ kurzer Zeit vollkommen beseitigt. Zur leichteren Orientierung der oben erwähnten Versuchsresultate sind hier einige Abbildungen beigefügt.

Zusammenfassung.

1. Der Nüchternwert des sog. geb. Blutzuckers wird durch Hunger von einwöchiger Dauer (Hunger von kurzer Dauer) nicht

Abb. 1. Hund Nr. 11.

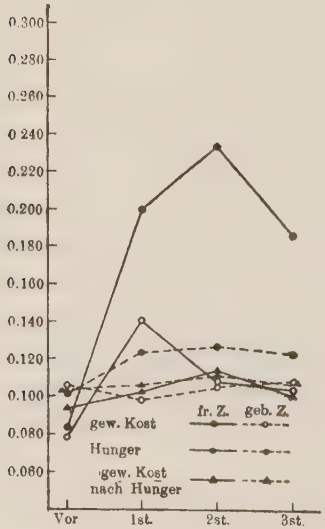
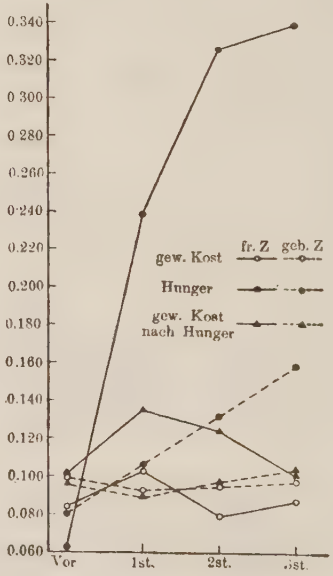


Abb. 2. Hund Nr. 16.



bedeutend beeinflusst, aber nach Hunger von dreiwöchiger Dauer (Hunger von langer Dauer) niedriger gefunden als sonst.

2. Durch Hunger steigt der Wert des sog geb. Blutzuckers nach Zuckerzufuhr an und die Steigerung desselben kommt bei langem Hunger sogar stärker zum Ausdruck, als bei kurzem.

3. Diese alimentäre Steigerung des sog. geb. Blutzuckers kehrt durch Wiederverabreichung kohlenhydratreicher gemischter Nahrung relativ in kurzer Zeit wieder auf den normalen Wert zurück.

4. Der höchste Punkt der Kurve des sog. geb. Blutzuckers nach Zuckerzufuhr fällt in der überwiegenden Mehrzahl der Fälle zeitlich mit dem des freien Blutzuckers zusammen.

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STUDIES ON A DIAMINOACID, CANAVANIN.

IV. The Constitution of Canavanin and Canalin.

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In a previous paper (1933), we made the assumption that canalin will be a kind of *o*-derivative of hydroxylamine and have a constitutional formula $CH_2(ONH_2)\cdot CH_2\cdot CH(NH_2)\cdot COOH$, because of the formation of γ-oxy-α-amino butyric acid from canalin liberating ammonia on hydrogenation and, on the other hand, no existence of hydroxyl group originally in the molecule of canalin. Accordingly we suggested in a previous paper (1933) that canavanin, which was considered as a kind of guanido derivative of canalin, should have a constitutional formula $NH_2\cdot C(NH)\cdot NH\cdot O\cdot CH_2\cdot CH_2\cdot CH(NH_2)\cdot COOH$, if canalin had the above formula.

In the present communication, we put forward our succession of the synthesis of canavanin by means of the condensation of α-benzoyl canalin with methylisourea by Kapfhammer's method (1934) and we, therefore, confirmed the constitution of canavanin as a guanido derivative of canalin previously assumed.

On the other hand, we prepared *o*-hydroxylamino acetic acid $CH_2(ONH_2)\cdot COOH$, which was formerly synthesized by Werner (1893), and compared its main properties due to the presence of NH_2O group with that of canalin; that is, firstly, the indication of Jeffe's reaction by picric acid and sodium hydroxide, secondly, the formation of oxy fatty acid liberating ammonia on hydrogeneration, and finally, the formation of the guanido derivative on guanidation, which gives the characteristic colour reaction of canavanin by the denatured nitroprussid. And the results showed that both substances indicated similar properties in these respects.

According to these results, we concluded canalin to be a kind of *o*-derivative of hydroxylamine and to have the formula $CH_2(ONH_2)\cdot CH_2\cdot CH(NH_2)\cdot COOH$ previously assumed.

Experimental.

I. Synthesis of canavanin.

Dibenzoylcanalin obtained by the usual method, was heated with a large quantity of 7% HCl solution at 100° in a short time. Then α-mono-benzoyl canalin was produced, liberating one molecule of benzoic acid. It melted at 150°, giving Jaffe's reaction, but not ninhydrin reaction, and it was soluble in aqueous mineral acid solution differing from that of dibenzoylcanalin.

Then excess of methylisourea was added to concentrated methyl alcoholic solution of α-mono-benzoyl canalin and the mixture was allowed to stand for more than two weeks. After that the reaction mixture was condensed *in vacuo* and, on adding ether to it, a viscous mass was separated, which was again dissolved in a small quantity of ethyl alcohol and, on cooling after adding butyl alcohol, the reaction product was crystallised out almost free from the remaining methylisourea.

The crystalline product was dissolved in water, and picric acid was added to the solution in order to separate the remaining α-monobenzoyl canalin as insoluble picrate.

The filtrate from the picrate contained mainly the guanidated substance, which gave the characteristic colour reaction for canavanin, but not ninhydrin reaction. From these facts we could presume the formation of α-benzoylcanavanin in this reaction.

Then the reaction product was heated with 10% HCl solution for twenty hours, the separated benzoic acid on cooling was removed, and the filtrate was evaporated *in vacuo* into dryness. Then it was again dissolved in water and flavianic acid was added to the aqueous solution, thus flavianate was crystallised out. It was identified with canavanin flavianate by the characteristic melting point, namely, that it sintered at 190° and melted at 215° under decomposition. The substance set free from the flavianate.

gave typical canavanin colour reaction by the denatured nitroprussid and ninhydrin reaction, and it also indicated the other properties shown by canavanin.

Thus we succeeded in the synthesis of canavanin from canalin by means of guanidation.

$$NH_2{\cdot}O{\cdot}CH_2{\cdot}CH_2{\cdot}CH(NHCOC_6H_5){\cdot}COOH + CH_3O{\cdot}C(NH)NH_2$$

α-benzoyl-canalin

$$= NH_2{\cdot}C(NH){\cdot}NH{\cdot}O{\cdot}CH_2{\cdot}CH_2{\cdot}CH(NHCOC_6H_5){\cdot}COOH + CH_3OH$$

$$NH_2{\cdot}C(NH){\cdot}NH{\cdot}O{\cdot}CH_2{\cdot}CH_2{\cdot}CH(NHCOC_6H_5){\cdot}COOH + H_2O$$

α-benzoyl-canavanin

$$= NH_2{\cdot}C(NH){\cdot}NH{\cdot}O_2CH_2{\cdot}CH(NH_2){\cdot}COOH + C_6H_5{\cdot}COOH$$

canavanin

II. The comparison of canalin with *o*-hydroxylamino acetic acid.

Synthesis of o-hydroxylamino acetic acid.

This acid was formerly obtained from benzenyl amidoxim acetic acid $C_6H_5C(NH_2):NO{\cdot}CH_2COOH$ by Werner (1893), now we could prepare it simply in another way; that is, at first benzhydroximic acid $C_6H_5C(OH){\cdot}NOH$ was condensed with brom acetic ether in presence of sodium carbonate in the alcoholic solution. Benzoylhydroxylamino acetic acid $C_6H_5CO{\cdot}NHO{\cdot}CH_2{\cdot}COOH$ thus obtained, was recrystallised from ethereal solution, melted at 139°–140° (Werner: 135°–138°) and showed the same properties as that obtained by Werner.

Then benzoyl hydroxylamino acetic acid was heated with 5% HCl solution at 100° for fifty minutes and benzoic acid separated on cooling, was removed. The filtrate from benzoic acid was evaporated *in vacuo* into dryness and dissolved in a small quantity of absolute alcohol, after adding ether to it, the solution was stood in a cold place. In this way, *o*-hydroxylamino acetic acid hydrochloride $CH_2(ONH_2){\cdot}COOH{\cdot}HCl$ was crystallised out in needles, which melted at 156° and were identified with that obtained by Werner (156°).

Comparison of canalin with o-hydroxylamino acetic acid.

o-hydroxylamino acetic acid does not reduce Fehling solution as in the case of canalin. When it was hydrogenated by means of platinum black, it was converted into glycollic acid liberating ammonia (I), as was the case with canalin (II).

(I) $CH_2(ONH_2)\cdot COOH + 2H = NH_3 + CH_2(OH)\cdot COOH$

(II) $CH_2(ONH_2)\cdot CH_2\cdot CH(NH_2)\cdot COOH + 2H$
$= NH_3 + CH_2(OH)\cdot CH_2\cdot CH(NH_2)\cdot COOH$

Previously (1935) we considered Jaffe's reaction indicated by canalin, to be due to the presence of NH_2O group in its molecule. Now the synthesized *o*-hydroxylamino acetic acid above obtained, also gave the distinct Jaffe's reaction. We tested this reaction on several derivatives of this acid and canalin. The results were as follows:—

	Jaffe's reaction.	Ninhydrin reaction.
$NH_2O\cdot CH_2\cdot COOH$ *o*-hydroxylamino acetic acid	+	−
$NH_2O\cdot CH_2\cdot CH_2\cdot CH(NH_2)\cdot COOH$ canalin	+	+
$NH_2O\cdot CH_2\cdot CH_2\cdot CH(NHCOC_6H_5)\cdot COOH$ α-benzoylcanalin	+	−
$NH_2O\cdot CH_2\cdot CH_2\cdot CH(OH)\cdot COOH$ γ-*o*-hydroxylamino-α-oxybutyric acid	+	−
NH_2OH hydroxylamine	+	−
$C_6H_5C(OH):NOH$ benzhydroximic acid	−	−
$C_6H_5CO\cdot NH\cdot O\cdot CH_2\cdot COOH$ benzoyl hydroxylamino acetic acid	−	−
$C_6H_5CO\cdot NH\cdot O\cdot CH_2\cdot CH_2\cdot CH(NHCOC_6H_5)COOH$ dibenzoyl canalin	−	−
$CH_3CH:N\cdot O\cdot CH_2\cdot CH_2\cdot CH(NH_2)\cdot COOH$ ethyliden canalin (1935)	−	+
$NH_2\cdot C(NH)NHO\cdot CH_2\cdot CH_2\cdot CH(NH_2)COOH$ canavanin	−	+

From these results, we know that *o*-derivatives of hydroxylamine ($NH_2\cdot O\cdot R$) gives Jaffe's reaction, but it is not with *N*-derivatives ($R\cdot NHOH$).

We considered previously (1932) that the characteristic ruby colouration given by canavanin with the denatured nitroprussid was due to the presence of urea giving group in its molecule, or to the presence of $NH_2\cdot C(NH)\cdot NH\cdot O$ group, if, therefore, the

guanido derivative of *o*-hydroxylamino acetic acid be obtained, which should contain $NH_2{\cdot}C(NH){\cdot}NH{\cdot}O$ group, it must give this reaction too. Ordinary guanido derivative of fatty acid, namely, arginine or glycocyamine, does not give canavanin reaction by the denatured nitroprussid in the neutral solution, but only in weak alkaline solution it indicates unstable red colour.

Accordingly we guanidated *o*-hydroxylamine acetic acid with methyl isourea by means of Kapfhammer's method (1934) and tested the guadniation product in this respect. The product thus prepared, which was considered to have the formula $NH_2{\cdot}C(NH)NHO{\cdot}CH_2{\cdot}COOH$, gave the typical canavanin reaction by the denatured nitroprussid. According to this fact, the characteristic colour reaction of canavanin was proved to be due to the presence of the group $NH_2{\cdot}C(NH){\cdot}NH{\cdot}O$ in its molecule.

In conclusion, we know that canalin is a kind of *o*-derivative of hydroxylamine, having the formula $NH_2{\cdot}O{\cdot}CH_2{\cdot}CH_2{\cdot}CH(NH_2){\cdot}COOH$, and canavanin is a kind a guanido derivative of canalin, having the formula $NH_2{\cdot}C(NH){\cdot}NH{\cdot}O{\cdot}CH_2{\cdot}CH_2{\cdot}CH(NH_2)COOH$.

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CAUSE OF THE POTENTIAL DRIFT OF QUINHYDRONE ELECTRODE APPLIED TO SOLUTIONS OF ALKALINE BUFFER, OF AMINO ACID OR OF PROTEIN, OR TO PLASMA.

(Studies on the blood pH estimated by the glass electrode method. IV)

By

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Introduction.

Since Biilman (1921) reported the potential of quinhydrone electrode as an indicator of hydrogen ion concentration, its applicability to blood and other biological materials has been discussed by many investigators. Now there is a general belief that the method is applicable to the pH-determination of plasma when the potential is measured immediately after the saturation of the plasma with quinhydrone. Actually the potential of the quinhydrone electrode changes with the lapse of time by some secondary reaction taking place in the plasma, so that the practical estimation of the equilibrated potential is attained before the change proceeds to interfere with it. As to the applicability of the method to the whole blood, however, there are some objections because of the instability of the potential.

In order to obtain a correct potential of the quinhydrone electrode applied to plasma, some investigators Cullen and Biilman (1925), Mislowitzer (1926), Schau-Kuang-Liu (1927), Mikawa (1933), etc. recommended that the potential be read within several ten seconds after adding quinhydrone to plasma, while others Laug (1930), Hanke (1930), etc. recommended that the potential be extrapolated to zero time from

observed data.

As to the course of the potential drift, results reported by previous investigators do not coincide well with one another. Kolthoff (1925), Mislowitzer (1926), Reimers (1929), Laug (1930), etc. found the change of the potential towards the acid side. But Cullen and Biilman (1925), Schau-Kuang-Liu (1927), Mori (1927), Mikawa (1933), etc. did not agree with it. According to Hanke (1931), the direction of the potential change depends upon the quantity of quinhydrone added to plasma.

As to the cause of this change, too, opinions of previous investigators differ from one another. Cullen and Biilman (1925) supposed it to be the effect of an oxidation-reduction system of an enzym or similar substances contained in plasma. Schau-Kuang-Liu (1927, 28) maintained that the cause is a sum of miscellaneous phenomena, such as the saturation process of quinhydrone, the effect of protein and dissolved oxygen, some complicated chemical reactions between serum and quinhydrone, and especially the oxidation of hydroquinone. Kolthoff (1925), Vellinger and Roche (1925), Mislowitzer (1928), Laug (1930) and Hanke (1931) attributed it to the effect of protein.

As to the protein error, Kolthoff (1925) found that the quinhydrone electrode method gave a smaller pH value for protein solutions than the hydrogen gas electrode method, and the potential of the quinhydrone electrode changed with the lapse of time towards the acid side, such an error depending upon the sort of protein, its concentration, and the pH of the solution. Linderström-Lang (1926), Vellinger and Roche (1925), and Reimers (1929) also ascertained the protein error. Reimers (1929) maintained that the potential read early enough after saturation with quinhydrone gave a correct pH value for a protein solution, and the cause of the so-called protein error was due to some chemical reaction between the protein and quinhydrone. He also found that the colour of the protein solution mixed with quinhydrone rapidly changed from brown to red, and he attributed this colour change to the existence of quinone.

Applicability of this electrode to amino acid solutions was

studied by Yasumaru (1931) and Hiraki (1932). Hiraki found that the potential changed with the lapse of time towards the acid side, when the pH of the solution was higher than ca. 5, and its initial potential (read ca. 1 minute after saturation with quinhydrone) gave a much smaller pH value than that determined by the hydrogen gas electrode method. Reimers (1929) observed a colour change in an amino acid solution mixed with quinhydrone and he attributed it to the existence of quinone.

In an alkaline solution of pH 8.5 or higher, the potential of the quinhydrone electrode gives smaller pH value than that obtained by the hydrogen gas electrode method. Thus Biilman (1927) marked the limit of applicability of the quinhydrone electrode at pH 8.5, when the solution has a strong buffer action. On the other hand, LaMer and Parsons (1923), Auerbach and Smolzik (1924), Kolthoff (1925), Rabinowitsch and Kargin (1927), Reimers (1929), Laug (1930), etc. found an error even in a scope of pH 5–8.5, mostly towards the acid side, but sometimes towards the alkaline side. This error is believed to be larger when the temperature is higher, or the buffer action of the solution is weaker, or its pH is higher.

Concerning its cause, LaMer and Parsons (1923) and Biilman (1927) maintained that the change of the pH of the solution was due to the acid dissociation and also to the autoxidation of hydroquinone, which caused the potential drift in the direction of more positive potentials (towards the side of smaller pH value) with the lapse of time. Kolthoff (1925) and Schau-Kuang-Liu (1928) agreed with them on the autoxidation of hydroquinone. On the other hand, Kolthoff and Bosch (1927), and Rabinowitsch and Kargin (1927) found that acid impurities contained in the quinhydrone sample made the pH of the solution decrease. Reimers (1929) insisted that those acid impurities contained in quinhydrone might cause a remarkable error.

As stated above, the quinhydrone method includes several sources of error, which have not yet been explained definitely. When a glass electrode is applied to the solution mixed with quinhydrone, the potential is not influenced by the oxidation-

reduction system of quinhydrone, so that the real pH value of the solution can be measured by this method, and thus it can be determined whether the error of the quinhydrone electrode, especially the potential drift, is due to the actual change in the pH of the solution, or to the change in the oxidation-reduction system contained in it. This is the aim of our present study.

Experimental Methods.

The materials used were McIlvaine's buffer and Sörensen's phosphate buffer, as well as aspartic acid, glycocoll, leucin, α-alanin, and blood albumin dissolved in these buffer solutions, and also plasma.

The method of measuring the potential with the glass electrode was the same as described in our first report (1935). The potential of the quinhydrone electrode was measured by immersing a gilded platinum electrode in a solution saturated with Kahlbaum's quinhydrone.

The temperature was 25°C throughout all experiments except when the effect of the temperature was being studied. Details of the experimental method will be related below in the corresponding sections.

Experimental Results and Discussions.

A. Experiments on Buffer Solutions

1. The potential drift of the quinhydrone electrode at a high pH.

In McIlvaine's buffer and Sörensen's phosphate mixture of different pH values, the potential of the quinhydrone electrode was measured repeatedly at various times after saturating it with quinhydrone. Results are shown in Table I. In its second column are the pH values of the buffer solutions which were determined by means of the hydrogen gas electrode, in the third column the pH values calculated from the potential of the quinhydrone electrode read at 1–5 minutes after the saturation of quinhydrone (we will call it the initial reading), and in the last column the difference

Table I.

Buffer solution	Hydrogen gas electrode	Quinhydrone electrode	
		Initial reading	Change after 60 minutes
McIlvaine's	2.812 pH	2.822 pH	−0.003 pH
„	4.821	4.825	+0.007
„	5.937	5.938	+0.003
„	6.999	6.983	+0.010
Phosphate	7.310	7.293	+0.017
„	7.848	7.824	+0.029

(in pH unit) between the initial reading and the reading after 60 minutes. The positive sign indicates an increase of the pH with the lapse of time, and the negative sign, its decrease. Here we see that, in the pH range studied (pH 3–8), the pH value calculated from the initial reading of the quinhydrone electrode coincides within ca. 0.02 pH with that found by the hydrogen gas electrode method. With a solution of pH 7 or less, the potential drift for one hour remained within ca. 0.01 in pH unit, i.e. within the scope of experimental error, but it showed a tendency of growing larger when the solution was more alkaline (e.g. a drift of 0.03 pH at pH 7.85). Contrary to LaMer and Parsons' results, the direction of this potential drift was towards a more negative potential (towards a larger pH value) in most cases.

In alkaline solutions, the colour changed with the lapse of time from light yellowish brown to reddish brown, and the higher the pH of the solution, the larger was the speed of the change (cf. Reimers, 1929).

Next, by means of the glass electrode, the pH drift was followed with the lapse of time on a phosphate solution of pH 7.85 saturated with quinhydrone, quinon or hydroquinone. Results are given in Table II, in which the time is that after the saturation, and the pH value at the zero time is that of the solution before the saturation. The potential drift of the quinhydrone electrode expressed by the pH unit is also given for comparisons sake. It can be seen in the table that the pH value of the solution calculated

Table II.

Time (minute)	Quinhydrone electrode	Glass electrode		
		+ Quinhydrone	+ Quinone	+ Hydroquinone
0		7.848 pH*		
1	7.824 pH	7.840	7.848 pH	7.602 pH
5	7.826	7.837	7.819	7.599
10	7.832	7.830	7.806	7.597
20	7.838	7.820	7.775	7.597
30	7.843	7.811	7.740	7.599

* pH without admixture of quinhydrone.

from the quinhydrone electrode potential increases gradually with the lapse of time, while the real pH value of the same solution saturated with quinhydrone, which was determined by means of the glass electrode, decreases gradually during the same period. Similar, but a more speedy decrease of the real pH value is observed in the same buffer solution saturated with quinone. When the solution is saturated with hydroquinone, its real pH value shows an initial shift towards the acid side, but remains constant thereafter. Thus the acid change of the quinhydrone solution with the lapse of time is mainly due to a certain chemical change of quinone. The initial shift caused by hydroquinone mentioned above, does not appear appreciably in it, as the real pH of the quinhydrone solution at 1 minute after the saturation with quinhydrone, is approximately the same as the pH of the solution before the saturation. The reason why the acid change is more remarkable in the saturated quinone solution than in the saturated quinhydrone solution is supposed to be that the concentration of quinone is higher in the former than in the latter, in which the quinone is not really saturated.

It was also found that the colour of the quinone solution changes with the lapse of time from deep yellow to dark brown, increasing the reddish tone, while the colour change of the hydroquinone solution is rather slight, changing from colourless to slightly reddish yellow. This indicates that the colour change of

the quinhydrone solution is mainly due to that of quinone as already found by Reimers (1929). According to LaMer and Rideal (1924), hydroquinone in alkaline solutions is oxidized to quinone by oxygen in the air, and the colour change of the solution may be due to the formation of complex-ion of hydroquinone as an intermediate product. In our opinion, however, a part of this colour change is due to the formation of quinone from hydroquinone.

Now we can explain the potential drift of the quinhydrone electrode as follows. It is an algebraical sum of the effect of a potential increase (a decrease in the pH value) and that of a potential decrease (an increase in the pH value). The former effect is mainly due to an acid substance produced by a certain chemical change of quinone, while the latter effect is mainly due to the diminution of the oxidant (quinone). Besides these, it is possible that this chemical product establishes an oxidation-reduction system which may affect the quinhydrone electrode potential. As we know nothing about it, however, it is put aside from the present discussion. The above explanation makes clear an incongruity existing between the direction of the potential drift found by LaMer and Parsons (1923), and that by the present writer. The details will, however, be discussed later in the section on the results with amino acids.

Since LaMer and Parsons (1923), the cause of the potential drift of the quinhydrone electrode has been explained as the autoxidation of hydroquinone. The reason why the initial reading of the quinhydrone electrode gives a correct pH has been understood as due to the slow speed of this autoxidation (cf. Biilman, 1927). But, according to our results, the effect of the autoxidation of hydroquinone in a saturated quinhydrone solution is negligible compared with the effect of the chemical change of quinone, so that the above explanation is not correct. That the initial reading of the quinhydrone electrode gives an approximately correct pH value may be explained by the counteraction of two oppositely directed influences upon the potential, i.e. the diminution of quinone and the production of an acid substance from it.

As to the chemical change of quinone, it is generally believed

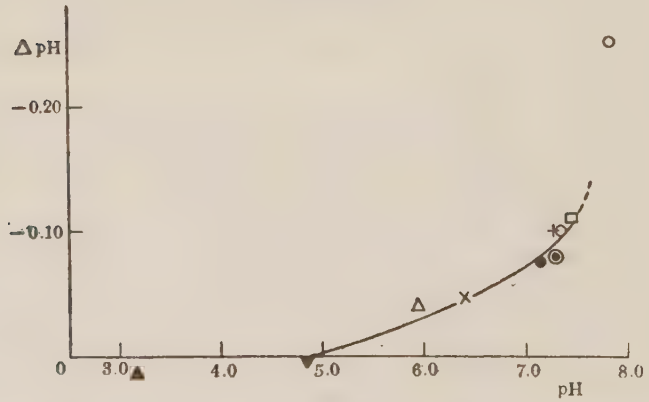
that quinone undergoes a remarkable autoxidation in an alkaline solution, producing a sort of oxyquinone which acts as an acid (cf. Beilstein, 1897). Thus it is natural that the reaction of a buffer saturated with quinone or quinhydrone shifts to the acid side with the lapse of time.

2. Cause of the acid shift occuring when the solution is saturated with hydroquinone.

As stated above, saturation with hydroquinone makes the pH of a buffer solution decrease at once, without, however, a further pH change. A similar pH shift is also seen in a solution of amino acid or of protein, or in plasma, when they are saturated with hydroquinone.

The shift of the pH (Δ pH) caused by the saturation with hydroquinone is studied in various solutions of different pH. Data observed are assembled in Table III and are plotted in Fig. 1.

Fig. 1.
pH change accompanied with the saturation of hydroquinone.



of which the ordinate gives the decrease of the pH ($-\Delta$ pH), and the abscissa the original pH of the solution (without hydroquinone). Here we see that the shift of the pH caused by the saturation of hydroquinone is within the scope of experimental error when the pH of a solution is lower than 5, but it is appre-

TABLE III.

pH of buffer solutions before as well as after saturation with hydroquinone (by glass electrode)

Solution	Marks in Fig. 1	Before addition	After addition	Difference (△ pH)
McIlvaine's buffer +1% glycocoll	▲	3.146 pH	3.158 pH	+0.012
McIlvaine's buffer +5% glycocoll	▼	4.831	4.835	+0.004
McIlvaine's buffer	△	5.937	5.895	−0.042
McIlvaine's buffer +1% aspartic acid	×	6.439	6.388	−0.051
Phosphate buffer +1% glycocoll	●	7.232	7.156	−0.076
Phosphate buffer +1% α-alanin	+	7.285	7.187	−0.098
Phosphate buffer	○	7.310	7.212	−0.098
Phosphate buffer +1% blood albumin (supernatant)	◉	7.302	7.222	−0.080
Plasma	□	7.455	7.345	−0.110
Phosphate buffer	○	7.848	7.599	−0.249

ciable in a solution of higher pH. The higher the original pH of the solution, the more remarkable the pH shift. All points on the figure tend to lie in a curve, despite the different natures of the constituents of the solution. This suggests that the acid shift is caused by an acid action of the hydroquinone sample. The fact that this acid shift takes place at once when hydroquinone is added to the solution, without being accompanied by a further pH change, supports this assumption. The incomplete formation of a smooth curve seems to be mainly due to the variety of the buffering actions of various solutions.

The sample of hydroquinone can cause an acid shift by two possible ways, i.e. by the acid dissociation of hydroquinone itself, and by some acid impurity contained in the sample. To check these two possibilities, the following calculations are carried out. As is given in Table III, we find an acid shift of 0.10 pH by saturating a M/15 phosphate solution of pH 7.31 with hydro-

quinone. Assuming the negative logarithm of the second dissociation constant of phosphoric acid as $pK_2=6.9$ (cf. Britton (1932) and also Shima's report which will soon appear elsewhere), the quantity of an acid substance necessary to cause an acid shift of 0.10 pH in the above mentioned phosphate solution is calculated to be 0.0033*N*. According to Vaubel (1899), the solubility of hydroquinone is 6.7% (≒6.5 M) at 20°C, and after Sheppard (1921), its pK_1 is 9.76 at 18°C. Assuming that these values are practically applicable at 25°C, we can calculate the quantity of hydroquinone dissociated at 7.21 (the pH after the acid shift) to be 0.0018 M. Therefore, about a half of the acid shift which is observed in the phosphate solution is due to the acid dissociation of hydroquinone, and the remaining half, corresponding to 0.0015*N* acid is due to acid impurity contained in the hydroquinone sample.

We can apply a similar calculation to an observation with McIlvaine's buffer of pH 5.94 (7.5 parts of 0.1 M citric acid + 12.5 parts of 0.2 M Na_2HPO_4), in which a shift of 0.04 pH is found on saturating it with hydroquinone. Assuming that pK_3 of citric acid is 5.49 after Hastings and Van Slyke (1922), and neglecting its second dissociation, we find that an acid shift of 0.04 pH is caused by 0.0018*N* acid of which 0.0001*N* is occupied by hydroquinone dissociated. Thus the remaining 0.0017*N* acid is due to the impurity. This figure almost coincides with the quantity of the impurity found in the above mentioned experiment with phosphate solution. Thus in a solution of a pH lower than 7, the acid shift caused by saturation with hydroquinone is mainly due to acid impurity contained in the sample of hydroquinone, while in a solution of higher pH, the acid dissociation of hydroquinone comes to play an important rôle. When the phosphate solution of pH 7.85 was saturated with quinhydrone, no remarkable pH shift was observed immediately after the saturation (cf. Table II). This might be partly due to the difference in the impurity contained in the sample used. The quinhydrone used was Kahlbaum's sample "for analytical purposes", while the hydroquinone was the "extra-pure" sample of Merck. The main reason, however, is supposed to be in the concentration of hydroquinone, which is

higher when hydroquinone is saturated than when quinhydrone is saturated in the solution.

3. Conclusions obtained from experiments on buffer solutions.

In an alkaline solution, a slight potential drift appears in the potential of the quinhydrone electrode. Its main cause is not in the autoxidation of hydroquinone as previously believed, but in the acid production due to the chemical change of quinone. Though the sample of hydroquinone can cause the acid shift of the solution by its own acid dissociation and also by acid impurity contained in it, the effect is negligible for a buffer, when it is saturated with a quinhydrone sample (Kahlbaum). Thus the quinhydrone electrode method includes various sources of error. This error grows with increase of the pH of the solution. But in a buffer solution (up to ca. pH 8, at least), the potential of the quinhydrone electrode read ca. 1 to 5 minutes after saturation with quinhydrone enables us to calculate from it the reliable pH value with an accuracy of 0.02 pH.

B. Experiments on Amino Acid, Protein and Plasma.

1. Potential drift of the quinhydrone electrode immersed in a solution of amino acid or of protein.

The potential of the quinhydrone electrode was repeatedly measured for a certain period at 25°C in McIlvaine's buffer solutions of various pH, in which 1% each of aspartic acid, α-alanin, leucin or glycocoll was dissolved. Results are given in Table IV, Nos. 1–4. In the first column is given the pH calculated from the potential of the quinhydrone electrode read ca. 1 minute after saturation with quinhydrone. This can be regarded as the approximately correct pH value of the solution (refer discussion below). In the second column is given the potential drift observed 30 minutes after the saturation, the value being represented by the pH unit. In all cases, this drift directs itself towards a higher pH value, which is indicated by the '+' sign

Table IV.

No. 1		No. 2		No. 3		No. 4		No. 5		No. 6	
1% aspartic acid		1% α-alanin		1% leucin		1% glycocoll		1% glycocoll		5% glycocoll	
25°C		25°C		25°C		25°C		18°C		18°C	
1 min.	30 min.	1 min.	30 min.	1 min.	30 min.	1 min.	30 min.	1 min.	30 min.	1 min.	30 min.
2.455 pH	+0.003 pH	2.962	+0.005	2.874	+0.009	2.998	+0.006	3.018	+0.002	3.477	+0.037
3.765	+0.002	3.582	+0.006	3.527	+0.009	3.592	+0.007	3.612	+0.009	3.909	+0.045
4.482	+0.031	4.792	+0.008	4.782	+0.013	4.797	+0.010	4.803	+0.011	4.812	+0.078
5.981	+0.059	6.311	+0.079	6.319	+0.128	6.300	+0.281	6.311	+0.130	6.199	+0.516
6.443	+0.124	7.175	+0.525	7.194	+0.792	7.187	+1.990	7.204	+0.406	7.138	+1.964

attached to the figure. The potential drift is within the scope of experimental error when the 1% amino acid solution has a pH of 5 or lower (4 or lower for the aspartic acid solution). The higher the pH of the solution, the more remarkable the potential drift. This potential drift accompanies a colour change of the solution towards a brownish red, which is more remarkable than the colour change observed in the solution without amino acid or protein.

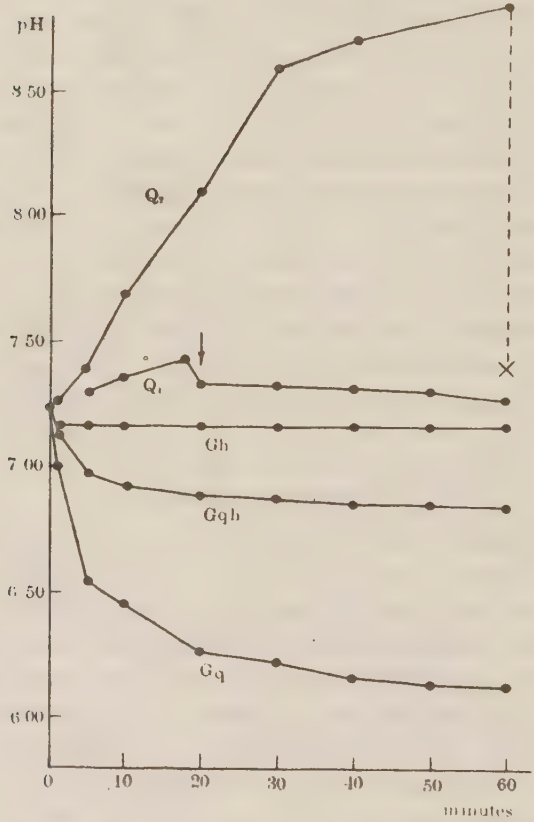
No. 5 of the table gives the results with 1% glycocoll solution at 18°C. Though these results agree with those at 25°C in that the potential change becomes notable from pH 6, the magnitude of the change is less than at 25°C. When the concentration of glycocoll increases to 5%, the change is notable even in a solution of pH 3.48 (cf. No. 6 of the table). Thus, the potential change of the quinhydrone electrode with the lapse of time grows as the temperature or the concentration increases. This suggests a certain chemical change as the cause of the potential drift stated here.

2. The pH change of a solution of amino acid or protein traced by means of the glass electrode.

1% glycocoll solutions of various pH values were prepared by dissolving glycocoll in McIlvaine's buffer solutions. After saturating them with quinhydrone, the pH values of the solutions were measured by means of the glass electrode repeatedly for a certain period. A remarkable change of the pH was observed in a solution of pH higher than 5, speed of the change increasing with the increase of pH of the solution. This fact coincides well with the result found with the quinhydrone electrode, but the direction of the change is opposite, i.e. the pH observed with the glass electrode changed towards lower pH, while the potential of quinhydrone electrode deviated towards higher pH.

In order to find the cause of this change, the following measurements were made. The same 1% glycocoll solutions of pH 7.23 were saturated with quinhydrone, quinone and hydroquinone respectively, and their pH changes were followed by means of the glass electrode for a certain period after the saturation. Results are shown by Fig. 2. The curve G_{qh} represents the results

Fig. 2.
Potential drift of quinhydrone electrode and pH change in 1% glycocoll solution saturated with quinhydrone, quinone or hydroquinone.



with quinhydrone, the curve G_q with quinone and the curve G_h with hydroquinone. For the sake of comparison, two examples of the quinhydrone electrode, Q_1 and Q_2, are also given in the figure, their potential being converted into the pH value. The starting point of the curve G_{qh} or G_q represents the pH of the glycocoll solution before addition of quinhydrone or quinone.

Results are similar to those found in alkaline buffer solutions. By means of the glass electrode, an acid change was observed with

quinhydrone, a more remarkable acid change with quinone and an acid shift without further change with hydroquinone. The magnitude of the acid change observed with quinhydrone or quinone was, however, much more remarkable in a glycocoll solution than in an alkaline buffer solution of the same pH. The colour change of quinone or hydroquinone was also more remarkable and the reddish tone was stronger in the former solution than in the latter. The colour change of hydroquinone was, however, much less than that of quinone.

Thus we see that the nature of the potential drifts observed in glycocoll solutions and in alkaline buffer solutions is the same. That is, the potential drift of the quinhydrone electrode in a glycocoll solution is due to a certain chemical change of quinone which produces an acid substance, while the autoxidation of hydroquinone as a cause of the drift is negligible. Addition of hydroquinone makes the pH of the solution shift towards lower pH by its own acid dissociation and by acid impurity contained in the sample, but it does not play a rôle in the potential drift discussed here.

The same results were observed with aspartic acid, α-alanin and leucin. In the supernatant liquid of 1% blood albumin solution, similar phenomenon was seen, though the pH change of it was slight because of its small concentration. Thus, as was maintained by Reimers (1929), Laug (1930) and Biilman (1927), the potential drift of the quinhydrone electrode in a protein solution is due to a certain chemical change between protein and quinhydrone.

3. Criticism of disparate results hitherto reported on the potential drift of the quinhydrone electrode.

As stated above, we found that the potential of the quinhydrone electrode changed with the lapse of time towards a higher pH value in amino acid solutions. In Yasumaru's report (1931), such a drift of the potential may also be observed. Hiraki (1932) reported, however, that the potential changes towards lower pH, when the pH of the solution is higher than 5.

To find the cause of this incongruity, we followed the potential drift of Hiraki's quinhydrone electrode in a 1% glycocoll solution of pH 7.23. This electrode was an injection syringe of which the inner plunger bore a platinum wire, which was stuck in a mud of quinhydrone sucked up into the syringe. The results are represented by Q_1 in Fig. 2. Q_2 in the same figure is the course of the potential drift of an ordinary quinhydrone electrode of which the electrode vessel was a small beaker of ca. 20 cc. capacity, and the electrode wire did not reach the mud of quinhydrone precipitated in the bottom of the vessel. We see that the potential drift of the ordinary type of electrode proceeds much more quickly than that of the syringe type.

In the upper portion of the amino acid solution saturated with quinhydrone, the concentration of quinone is supposed to be less than in its lower portion, because the loss of quinone caused by its chemical change with amino acid is restored only by a dissolution of the quinhydrone mud in the bottom of the vessel. Therefore, the acid change may be less, but the excess of reductant may be larger, in the upper portion than in the lower. This is supposed to be the reason, why the potential changes markedly towards the side of higher pH in the ordinary type of electrode. When the electrode vessel is shaken vigorously to distribute the mud of quinhydrone uniformly in the solution, the electrode potential increases immediately. This is shown by a sudden decrease of pH value at a point of 60 minutes in the curve Q_2 (shown by '×' sign in the figure). The fact coincides with the above mentioned supposition. On the contrary, the electrode wire of the syringe type of vessel is subjected to more of the acid production of quinone which acts to cancel the effect of the excess of reductant, so that the potential drift is rather small in the curve Q_1. Here we see that the potential which is changing towards higher pH shifts at once towards lower pH when the electrode wire is stuck suddenly in the mud at a point of ca. 20 minutes (indicated by an arrow), and thereafter the potential goes, though slowly, towards the same direction. These results correspond to Hiraki's.

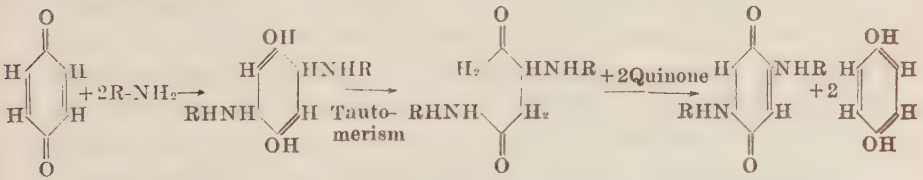
In our experiments, the potential of the quinhydrone electrode

decreased with the lapse of time in a protein solution, while Kolthoff (1925) and others observed the reverse. Such an incongruity was also found in alkaline buffer solutions. This may also be explained in a similar manner.

4. Cause of the pH change in a solution of amino acid or of protein saturated with quinone.

The pH change of an amino acid or protein solution saturated with quinone mentioned above may partly be due to autoxidation of quinone as was discussed above in reference to alkaline buffer solutions. But the change is so remarkable in the former solution that it can not be explained by the autoxidation of quinone alone, and thus a special chemical reaction may be supposed between quinone and amino acid or protein. An intensive reddish colour of the amino acid or protein solution saturated with quinhydrone or quinone supports this view.

It is widely believed that amino acid reacts with quinone to form a di-anilido compound. Refer to Hofmann (1863), Ville and Astre (1895), Posner (1904), Fischer and Schrader (1910), Siegmond (1910), Suida (1913), Suchanek (1914), etc. Posner explained the reaction as follows. First, quinone forms an addition-compound with amino acid ($R\text{-}NH_2$), then this compound undergoes a tautomerism, and at last it is oxidized by an excess of quinone to decompose into a dianilido-quinone and hydroquinone.



According to Suida, a similar reaction occurs in protein solutions.

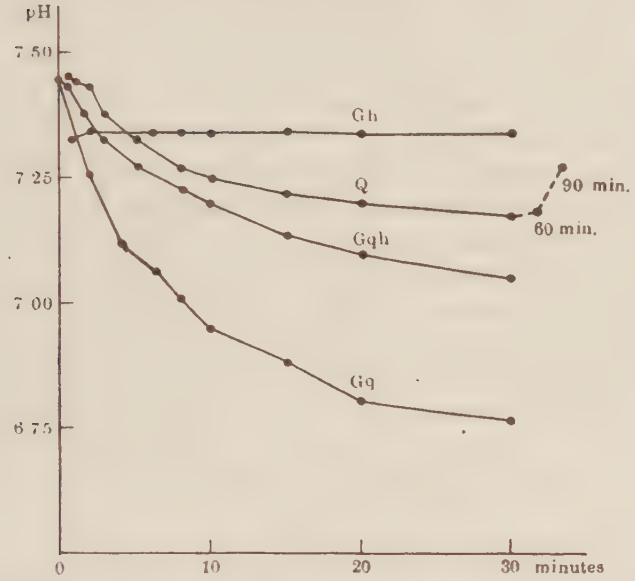
In every stage of the above reaction, the formation of an acid is possible, as the amino group of the amino acid is neutralized with quinone. Hydroquinone which is produced at the end of the

reaction also acts as an acid. On the other hand, this hydroquinone affects the potential of the quinhydrone electrode as an excess of the reductant. Tautomerism may account for the colour change. Thus the scheme of the reaction well explains the experimental results. From the chemical constitution of di-anilido-quinone, it is expected to affect the potential drift as a new oxidation-reduction system. As to the acceleration of the chemical change of quinone in an alkaline solution, no definite explanation can be given, except an effect of the acid dissociation of the products. In Table IV, it is seen that, in a 5% glycocoll solution, the potential drift is noticed at pH 3.48 and above, while, in 1% solution of glycocoll, α-alanin or leucin, it is seen at a pH above 6, an approximate isoelectric point of these amino acids. Therefore this chemical reaction between amino acid and quinone has no direct bearing upon the acid dissociation of the amino acid (in the sense of the classical theory of dissociation of amino acid).

5. *On the pH of plasma measured by means of the quinhydrone electrode.*

It is believed that the blood plasma contains ca. 7% protein and a minute quantity of amino acids. Therefore the cause of the potential drift of the quinhydrone electrode is supposed to be the same as that in the protein solution, while the effect of amino acids in it is negligible. To confirm this supposition, the potential drift of the quinhydrone electrode immersed in an oxalated horse plasma was followed for a certain period. On the other hand, the pH change of the same plasma was followed for the same period by means of a glass electrode, after the plasma was saturated with quinhydrone, quinone or hydroquinone. To saturate the plasma with the chemical without any loss of CO_2, one volume plasma was mixed under a liquid paraffin layer with ¼ volume solution of 1% NaCl containing a mud of the chemical. Results are shown in Fig. 3 in which the signs Q, G_{qh}, G_q and G_h has the same meaning as in Fig. 2. The directions of the pH change are the same in general as those obtained in a solution of amino acid or protein. Thus the main cause of the potential drift of quin-

Fig. 3.
Potential drift of quinhydrone electrode and pH change in plasma saturated with quinhydrone, quinone or hydroquinone.



hydrone electrode is a certain chemical change of quinone which produces an acid substance. That is, the potential of quinhydrone electrode is determined by an algebraical sum of a potential increase due to acid production and a potential decrease due to loss of the oxidant. Explanations of Cullen and Biilman(1925), and also of Schau-Kuang-Liu (1928) sketched in the introduction are invalid.

The direction and course of the potential change of the quinhydrone electrode were found to be different according to the case. The curve Q in Fig. 3 represents only one case. Such a variety of the potential course was a source of discussion among many former investigators, but it seems to us that the question can be solved by the explanation similar to that given for amino acid solution. The fact found by Hanke (1931), that the potential changes towards lower pH when an abundant quantity of quinhydrone is added,

while it changes towards the opposite direction when the quantity of quinhydrone is little, supports this explanation.

6. *Comparison between the pH value determined by means of the glass electrode and that calculated from the initial reading of the quinhydrone electrode.*

It is generally believed that, though the potential of a quinhydrone electrode immersed in a protein solution or plasma changes with the lapse of time, its value read shortly after saturation with quinhydrone enables us to calculate an approximately correct pH value of it. To confirm this fact in amino acid or protein solution, or in plasma, we calculated the pH value from the initial reading of the quinhydrone electrode applied to these solutions. In the solution of amino acid or protein, the reading was taken ca. 1 minute after saturation with quinhydrone. In plasma, it was taken 30 seconds after the saturation, because the potential drifted rapidly in this case. This value was compared with the pH value of the solution (without quinhydrone) determined by means of a glass

Table V.

Solution	Glass electrode	Quinhydrone electrode	
		Initial reading	By the extrapolation
McIlvaine's buffer +1% aspartic acid	2.465 pH	2.455 pH	2.455 pH
„	3.775	3.765	3.765
„	4.474	4.482	4.480
„	6.003	5.994	5.987
„	6.439	6.443	6.433
McIlvaine's buffer +1% glycocoll	3.146	3.152	3.152
Phosphate buffer +1% glycocoll	7.232	7.247	7.220
„	7.682	7.618	7.625
Phosphate buffer +1% blood albumin (supernatant)	7.302	7.304	7.299
Plasma	7.455	7.446	7.477

electrode. Results are shown in Table V. Here we see that the pH value calculated from the initial reading of the quinhydrone electrode coincides with that determined by the glass electrode method within 0.02 pH in 1% amino acid solution of pH 7.23 or lower, a dilute blood albumine solution of pH 7.30 as well as plasma. But in 1% glycocoll solution of pH 7.68, the former pH values are less by ca. 0.06 pH than the latter pH. This error is probably due to a rapid acid change of the amino acid solution saturated with quinhydrone. The cause of the so-called protein error reported by Kolthoff(1925), Linderström-Lang(1926), etc. is probably due to the same fact.

Some investigators recommended that the pH value be calculated from the potential of the quinhydrone electrode which is found by extrapolating the potential-time curve to the zero time. A coincidence of the value calculated by this method with that obtained from the initial reading shows us that this method is superfluous (cf. Table V).

The reason why the quinhydrone electrode gives an approximately correct potential for an instant immediately after saturation with quinhydrone is not to be explained by the slow speed of the chemical change, but by the fact that two oppositely directed effects of the chemical reaction of quinone with amino acid or protein approximately cancel each other. In an example with 1% glycocoll solution, given in Table V, the initial reading of the quinhydrone electrode gives a correct pH 7.25, which coincides with the pH of the solution without quinhydrone, 7.23, determined by the glass electrode method. The pH of the same solution saturated with quinhydrone is found to be 7.11 by the glass electrode at ca. 1 minute after the saturation. This supports the above explanation.

7. Conclusions obtained from experiments on amino acid, protein and plasma.

The so-called protein error and the potential drift of the quinhydrone electrode found in a solution of amino acid or protein, or in plasma are explained by the chemical change of quinone

which produces acid substances, probably a di-anilido-quinone and hydroquinone. This change becomes remarkable as the temperature, the concentration of the material, or the pH of the solution increases. In spite of this chemical change, a reliable pH value of a solution of amino acid or of protein can be obtained accurately to 0.02 pH, if it is calculated from an initial potential read within 1 minute after the saturation with quinhydrone, so far as the concentration of the amino acid or protein is not high and the reaction is not far alkaline. For plasma, the initial reading should be taken within 30 seconds, because of its rapid potential drift.

General Conclusions.

1. The potential drift of the quinhydrone electrode in amino acid solution, protein solution, or plasma is mainly due to a chemical change of quinone which produces acid substances, probably a di-anilido-quinone and hydroquinone. The protein error of the quinhydrone electrode is also due to the same. This chemical change is accelerated with the concentration of amino acid or protein, the pH value of the solution, or the temperature.

2. In an alkaline buffer solution, a similar acid change of quinone as above takes place, though to a less extent, probably because of its autoxidation.

3. Hydroquinone may undergo autoxidation in alkaline reaction, changing to quinone, as was reported by LaMer and his collaborators. But, as the change is rather slight, it is by no means the main cause of the potential drift of the quinhydrone electrode.

4. Saturation of a solution with hydroquinone causes an acid shift in a buffer solution of a pH higher than 5 by its own acid dissociation as well as acid impurity which may be contained in the sample. Such an acid shift, however, does not take place on saturating the solution with pure quinhydrone, so far as the reaction of the solution is not far alkaline, because the concentration of hydroquinone is rather low in the solution saturated with quinhydrone.

5. Though the quinhydrone electrode method includes various sources of error for measuring the pH of a solution, a reliable

value can be calculated accurately within 0.02 pH from the initial reading of the potential. For a buffer solution of up to pH 8, a reading taken within 5 minutes after saturation with quinhydrone will suffice. For a solution of amino acid or of protein, the reading taken within 1 minute will do, provided that the concentration of the solution is not high and its reaction is not far alkaline. For plasma, the initial reading must be taken within 30 seconds.

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ÜBER DIE BESTIMMUNG DES CHOLINS.

Von

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Vorwort.

Um die Spaltung des Lecithins durch das Ferment zu untersuchen, ist die Bestimmung der aus dem Lecithin ausgeschiedenen, zu Lecithin spezifischen Substanz, des Cholins nötig. Es gibt zwei Arten der Bestimmungsmethode, die biologische und die gravimetrische. Bei der letzten benutzt man die Tatsache, dass die verschiedenen Verbindungen des Cholins, je nach deren Art, in verschiedenen Lösungsmitteln schwer löslich sind.

Um die Spaltung der Phosphatide durch das Ferment zu erforschen, haben wir eine Untersuchung der gravimetrischen Bestimmungsmethode des Cholins begonnen, und wollen nun hier die Resultate veröffentlichen.

Experiment.

I. Die Bestimmungsmethode des Cholins durch reinalkoholisch gesättigte Sublimatlösung.

Wenn man der Alkohollösung des Cholins reinalkoholisch gesättigtes Sublimat beimischt, zeigt sich selbst bei einer Konzentration von 1:2000000 eine Fällung des Cholin-Sublimats und sie ist auch im Äther unlöslich; diese Tatsache wurde häufig zur Bestimmung des Cholins gebraucht. (Rosenthaler: Der Nachweis organischer Verbindungen. Ausgewählte Reaktion und Verfahren. Seite 527, 1914). Wir zentrifugierten die durch Beimischung von reinalkoholisch gesättigtem Sublimat zur Alkohollösung des Cholins entstandene weisse Fällung, entfernten die obere klare Flüssigkeit,

und nachdem die Fällung zweimal mit reinem Alkohol und zweimal mit Äther gewaschen war, wurde sie in warmem Wasser aufgelöst, der Stickstoff bestimmt (Mikrokjeldahl), und daraus die Cholinmenge berechnet.

1) 2% reinalkoholische Cholinchloridlösung wurde in ein Zentrifugierspitzglas getan und solange reinalkoholisch gesättigte Sublimatlösung beigefügt, bis keine Fällung mehr auftreten konnte, 10 Min. mit 3000 Sekundendrehungen zentrifugiert, die obere klare Flüssigkeit entfernt, die Fällung mit Alkohol und Äther gespült; nach jeder Waschung wurde zentrifugiert und die obere Flüssigkeit entfernt. Dieses Verfahren wiederholte man zweimal. Dann wurde der Fällung 5–8 ccm destilliertes Wasser beigemischt, erwärmt, vermengt und aufgelöst. Die Lösung wurde dann in den Microkjeldahlkolben umgefüllt, das Spitzglas dreimal mit warmem Wasser gewaschen und das Waschwasser auch in den Kolben getan. Dann wurde der Stickstoffgehalt des Kolbeninhalts festgestellt (Mikrokjeldahl) und aus diesem die Cholinchloridmenge bestimmt. D.h., da Cholinchlorid ($C_5H_{14}NOCl$) ein Stickstoffatom enthält, kann man nach folgender Formel die Menge des Cholinchlorids berechnen:

$$\frac{\text{N-Menge mal } 139{,}58}{14{,}008} = \text{Stickstoffmenge mal } 9{,}964 \text{ mg}$$

Tabelle I.

2% HCl-Cholin (ccm)	Gefundene N-Menge (mg)	Errechnete HCl-Cholin (mg)
0,5	0,925	9,213
0,5	0,901	8,976
0,5	0,905	9,014
0,5	0,899	8,954
0,5	0,901	8,976
Mittelwert	0,906	9,027

Wie das Resultat der ersten Tabelle zeigt, konnte man 0,906 mg Stickstoff, also 9,027 mg Cholinchlorid bestimmen; auf 10,0 mg

rechnet man durchschnittlich mit 90,27%. Das Resultat ist ungefähr zufriedenstellend.

2) Als nächstes Bestimmungsmaterial gebrauchten wir die Mischung gleicher Menge von je 2% reinalkoholischer Lecithin- und Cholin-Chlorid-Lösung, darauf fügten wir reinalkoholisch gesättigtes Sublimat hinzu und erzielten dadurch die Fällung des Cholin-Sublimats. Dann wurde nach der oben beschriebenen Methode die Cholinmenge bestimmt.

Tabelle II.

Gemischte Lösung des Lecithins u. HCl-Cholins (ccm)	Gefundene *N*-Menge (mg)	Errechnetes HCl-Cholin (mg)
1	0,735	7,322
1	0,588	5,863
1	0,630	6,282
1	0,534	5,323
1	0,370	3,685
Mittelwert	0,571	5,695

Als man dem Bestimmungsmaterial reinalkoholisch gesättigtes Sublimat beifügte, bildete sich gelblich-weisse klebrige Fällung. Nachdem diese zweimal mit Alkohol gewaschen worden war, zeigte sich eine deutliche Abnahme der Menge und nach zwei Ätherwaschungen war diese noch deutlicher; die Menge betrug, verglichen mit der vor dem Waschen, etwas weniger als 2/3. In dem Fall, in welchem man den Niederschlag sowohl mit Alkohol wie auch mit Äther gewaschen hatte, blieb nach Zentrifugieren von 10 Min. mit 3000 Sekundendrehungen eine gelblich-weisse Trübung der oberen Flüssigkeit wie auch des Filtrats. Die Fällung wurde dann in warmen Wasser aufgelöst, der Stickstoff festgestellt und die Menge des Cholin-Chlorids bestimmt.

In diesem Fall gewinnt man von 10,0 mg 5,695 mg also 57%. Wenn man dieses Resultat mit dem durchschnittlichen Wert von 90,27% beim Fall der nur Cholin-Chlorid-Lösung allein vergleicht, so ist der Wert bedeutend geringer. Hierauf wurde die Fällung

fünfmal in reinem Alkohol und fünfmal in Äther gewaschen, wobei man in der letzten Waschflüssigkeit immer noch Phosphor bemerken konnte. Dies bedeutet, dass Lecithin der Fällung des Cholin-Sublimat-Doppelsalzes beigemengt wurde. Man muss annehmen, dass die Trübung der Waschflüssigkeit dadurch hervorgerufen wurde, dass das der Fällung beigemischte Lecithin sich beim Waschen derselben in der oberen Flüssigkeit auflöste und zu gleicher Zeit das Cholin-Sublimat-Doppelsalz in der letzteren schwamm.

Wenn man ferner die Fällung, die aus Lecithin und Sublimat entstanden ist, in Chloroform auflöst, geschieht das schneller als bei Äther. Als man jedoch der Fällung des Cholin-Sublimat-Doppelsalzes Chloroform beigab, schüttelte und nach dem Zentrifugieren das Vorhandensein des Stickstoffes in der oberen Flüssigkeit nachweisen wollte, war das Resultat negativ, und die Folge der Experimente war kaum verschieden von der mit Äther.

II. Die Bestimmung des Cholins durch reinalkoholische Platin-Chlorid-Lösung.

Die Tatsache, dass das Cholin-Platinchlorid-Doppelsalz in reinem Alkohol, Äther, Chloroform unlöslich ist, dass es zwei Kristallisierungsformen gibt, und dass diese wiederum kombiniert sein können (Würfel und Oktaeder), ferner, dass bei Mischung von reinalkoholisch gesättigter Platin-Chlorid-Lösung und reinalkoholischer Cholinlösung, selbst bei einer Konzentration von 1:2000000 Fällung des Cholin-Platin-Chlorid-Doppelsalzes auftritt, wird, wie auch beim Sublimat-Doppelsalz, bei der Scheidung und dem Nachweis des Cholins ausgenützt. Wir haben auf diesem Gebiet folgende Experimente gemacht:

Als Bestimmungsmaterial wurde 0,1% und 2% reinalkoholische Cholin-Chlorid-Lösung gebraucht, diese in ein Zentrifugierspitzglas gefüllt, 5% reinalkoholische Platin-Chlorid-Lösung dazu getan, bis keine Fällung mehr möglich war, dann 10 Min. bei 3000 Sekundendrehungen zentrifugiert, die Fällung mit reinem Alkohol und Äther (oder Chloroform) gespült und wieder zentrifugiert und dies dreimal wiederholt. Die Fällung wurde auf dem Wasser-

bad erwärmt, mit einem Glasstäbchen zerrührt und aufgelöst, dann in einen Veraschungskolben umgefüllt, das Spitzglas dreimal gewaschen und das Waschwasser auch in den Kolben gegossen. Hierauf wurde die Stickstoffmenge bestimmt (Microkjeldahl) und daraus das Cholin berechnet.

Tabelle III.

Bestimmungsmaterial (ccm)	Gefundene N-Menge (mg)	Errechnete HCl-Cholin (mg)
0,1% HCl-Cholin 3 ccm = 3 mg HCl-Cholin	0,280	2,792
„	0,273	2,722
„	0,287	2,862
„	0,280	2,792
„	0,259	2,583
2% HCl-Cholin 1 ccm = 20 mg HCl-Cholin	1,961	19,544
„	1,891	18,846
„	1,681	16,752
„	1,821	18,148
„	1,751	17,451
„	1,751	17,451
2% HCl-Cholin 0,5 ccm = 10 mg HCl-Cholin	0,771	7,678
„	0,911	9,074
„	0,869	8,655
2% HCl-Cholin 0,1 ccm = 2 mg HCl-Cholin	0,168	1,675
„	0,182	1,815
„	0,175	1,745
„	0,179	1,787

Wie die Tabelle III zeigt, sind die Resultate dieser Experimente, wie folgt: die höchste Differenz ist 23,3%, die durchschnittliche 11,3% und, verglichen mit den vorigen Experimenten mit der Sublimatlösung, ist kein grosser Unterschied zu verzeichnen.

Als nächstes wurde nur reinalkoholische Lecithinlösung verwendet, der man 5% reinalkoholische Platin-Chlorid-Lösung

beimischte, bis keine Fällung mehr möglich war. Nachdem die gelbliche klebrige Fällung zentrifugiert war, konnte man in der oberen Flüssigkeit, die ganz klar war, auch Stickstoff und Phosphor deutlich erkennen. Die Fällung ist in Äther, und noch schneller in Chloroform löslich. Wenn man sie in Wasser tat, wurde sie, genau so wie das Lecithin allein, nach dem Schütteln und Zerrühren eine dickliche Flüssigkeit.

Dann fügte man zur Mischung von 2% reinalkoholischer Cholin-Chlorid-Lösung und 2% reinalkoholischer Lecithinlösung 4 ccm 5% reinalkoholische Platin-Chlorid-Lösung hinzu, bis eine weitere Fällung nicht mehr möglich war. Nach dem Zentrifugieren konnte man an 4 ccm oberer Flüssigkeit 0,56 mg Stickstoff feststellen. Die Fällung war hellgelb und klebrig und blieb leicht an der Glaswand hängen. Als man mit reinem Alkohol, Äther oder Chloroform spülte, wurde die Menge deutlich weniger; und je öfter man spülte, desto weniger wurde sie, schliesslich verschwand sie ganz. Die Waschflüssigkeit trübte sich gelblich-weiss und beim Zentrifugieren von 10 Min. mit 3000 oder selbst bei 20 Min. mit 4000 Sekundendrehungen wurde die obere Flüssigkeit nicht klar.

Da bei diesem Experiment die oben genannten Schwierigkeiten vorhanden sind, ist sein Resultat, wie die vierte Tabelle zeigt, folgendes: die durchschnittliche gefundene Menge war auf 10 mg 50,06%.

Tabelle IV.

Gemischte Lösung des Lecithins u. HCl-Cholins (ccm)	Gefundene *N*-Menge (mg)	Errechnete HCl-Cholin (mg)
1	0,612	6,102
1	0,460	4,886
1	0,309	3,071
1	0,631	6,282
1	0,743	7,399
1	0,266	2,653
Mittelwert		5,065

Es ist schwer aus der Fällung, die aus der Beimischung von reinalkoholischer Platin-Chlorid-Lösung zur gemischten Lösung von Cholin und Lecithin entstanden war, je nach Gebrauch von Chloroform, Äther oder Wasser, Cholin-Platinat zu scheiden oder Lecithin und Lecithin-Platinat zu entfernen.

III. Nachprüfung der Levene und Ingvaldsen-Methode.

Tierfelder und Schulze (1905) benutzten die verschiedenen Eigenschaften, welche dit Salzsäuresalze des Cholins und Colamins auf das Calciumoxyd haben, fügten der Chloridmixtur der beiden Basen Calciumoxyd zu, extrahierten alles im Soxhlet-Apparat mit Äther, gaben dem Ätherextrakt ätherische Picrolonsäure-Lösung bei, machten das durch Calciumoxyd befreite Colamin zu Picrolonat und lösten dies in Äther auf. Da bei Cholin-Chlorid mit dieser Methode das Cholin nicht frei gemacht wird, und es auch in Äther schwer löslich ist, extrahierten sie das Cholin-Chlorid durch Alkohol aus dem Satz, aus welchem Colamin-Picrolonat extrahiert wurde, und fällten das Cholin des Extraktes aus Sublimatsalz.

Levene und Invaldsen (1920) gebrauchten, da die Extraktion mit Äther im Soxhlet-Apparat zulange dauert, nicht diese Behandlung, sondern extrahierten einfach mit kochendem Aceton und, statt das Cholin durch Sublimat zu fällen, nahmen sie Pikrinsäure oder Platin-Chlorid.

Da wir wissen wollten, ob das Cholin durch Selbstspaltung vom Lecithin geschieden wird und ob man mit dieser Methode Cholin und Colamin einzeln bestimmen kann, haben wir nach der Levene- und Ingvaldsen-Methode folgende Experimente ausgeführt:

Wir gaben in den Meyerkolben 10 g altes Lecithin, mischten diesem 15 g Calciumoxyd bei, zerrührten die Mischung gut, brachten einen Rücksflusskühler an und extrahierten es im Wasserbad mit 200 ccm kochendem Aceton. Hierauf filtrierten wir den ganzen Extrakt, machten das Filtrat salzsäuresauer, liessen im Vakuum das Aceton verdampfen, fügten dem Rückstand eine kleine Menge destilliertes Wasser hinzu und filtrierten es. Das Wasser dieses Filtrats liessen wir verdampfen, gaben dem Rück-

stand wieder Calciumoxyd bei und extrahierten mit kochendem Aceton; diesen Vorgang wiederholten wir dreimal. Der ganze Extrakt wurde dann filtriert, das Filtrat eine Nacht in der Eiskammer stehen gelassen und, nachdem es am nächsten Tag noch einmal filtriert war, liess man das Wasser im Vakuum verdampfen. Dem Rückstand wurde etwas Wasser zugefügt; die Flüssigkeit wurde gut geschüttelt und filtriert. Das klebrige, farblose und durchsichtige Filtrat wurde mit Wasser zu 30 ccm aufgefüllt, und wir haben mit dieser Lösung folgende Versuche gemacht:

Man säuerte 6 ccm Filtrat mit Salzsäure, mischte 0,1 ccm wässerige Goldchloridlösung bei und liess die Mischung im Exsikkator auf Schwefelsäure stehen. 1 ccm wässerigen Goldchloridlösung enthält 6,827 mg $AuCl_4$. Die Mixtur stand 7 Tage im Exsikkator und, so oft man nachsah, konnte man kein Cholin-Goldchlorid-Kristall bemerken, sondern nur das des Goldchlorids. Wir wollten den Stickstoff des Kristalls nachweisen, doch gelang dies nicht. Das Cholin-Goldchlorid-Doppelsalz lässt sich in kaltem Wasser schwer auflösen und, da es sich in Wasser von 21° 75,2:1 auflöst, kann man mit Goldschlorid das Cholin in der wässerigen Lösung quantitativ nicht fällen.

Als man den Stickstoff von 1 ccm Filtrat (Microkjeldahl) feststellte, entwickelte sich Ammoniak, welches $n/100\,H_2SO_4$ 2,5 ccm neutralisierte; somit war 2,5 mal 0,140=0,350 mg Stickstoff vorhanden. Die Amino-Stickstoffmenge von 1 ccm Filtrat wurde nach Van Slyke'scher Methode festgestellt. Bei einer Zimmertemperatur von 22° und einm Luftdruck von 756 mm betrug die aus 1 ccm Filtrat entwickelte Stickstoff-Gasmenge 0,7 ccm und, wenn man die 0,2 ccm der Leerversuche davon abzieht, bleibt 0,5 ccm; d.h. die Aminostickstoffmenge beträgt 0,280 mg. Da man bei der Auswahl der zu diesem Versuch nötigen Reagenzien bes. des Natrium-Nitrits sehr vorsichtig sein muss, haben wir die verschiedenen Präparate zu den Leerversuchen benutzt und, wie die Tabelle zeigt, gebrauchten wir Präparat C, also das von Ishizu.

Das als Bestimmungsmaterial gebrauchte Lecithin ist Ovo-Lecithin von Merck; nach Hirano (1927) betrug das Verhältnis des Gesamtstickstoffs und des Aminostickstoffs dieses Präparats

Tabelle V.

Nr. des Experiments	Volumen des entwickelten Gases beim Blindversuch			
	A	B	C	D
1	0,31	1,10	0,19	0,39
2	0,21	0,12	0,20	0,22
3	0,30	0,30	0,20	0,23
4	0,12	0,62	0,20	0,20
5	1,10	0,30	0,22	0,18
6	1,20	—	0,22	0,22
7	0,80	—	—	0,28
8	—	—	—	0,21
Mittelwert	—	—	0,205	0,24

49/100, und nach Sakakibara enthält es 12% Kephalin.

Wie oben erwähnt wird, ist die Gesamt-Stickstoffmenge von 1 ccm Filtrat 0,350 mg und, da die Aminostickstoffmenge von 1 ccm 0,28 mg ist, ist Amino-N/Gesamt-N = 0,280 × 100/0,350 = 80,0%. Also muss man annehmen, dass die übrigen 20% vom Cholin herkommen. Darum geht es schwer, Cholin und Colamin durch diese Methode vollständig zu trennen.

Um aus den 12 ccm Filtrat die Salzsäure zu entfernen, fügte man Silberoxyd hinzu und filtrierte. Um den Kalk zu fällen, führte man in das Filtrat Kohlenoxyd ein und filtrierte. Das Filtrat wurde im Vakuum verdampft und auf 2 ccm konzentriert. Dann gab man 1 ccm alkoholisch gesättigte Picrinsäurelösung bei, stellte die Mischung in der Eiskammer kalt; am nächsten Tag zeigte sich gelblicher Kristall und, mikroskopisch besehen, war dieses nur Picrinsäure-Kristall. Hierauf mischte man 1 ccm verdünnte Salzsäure bei und brachte das Ganze in den Exsikkator bis zum nächsten Tag, doch konnte man auch dann kein Cholin-Picrat-Kristall feststellen. Deshalb wurde im Filtrat nicht so viel Cholin enthalten, dass man es als Picrat bestimmen konnte.

Diesen Versuchen nach kann man annehmen, dass das Cholin sich aus alten Lecithin durch Selbstspaltung scheiden lässt, und

konnte man die Stickstoffmenge feststellen, jedoch die Bestimmung des Cholins mit verschiedenen Reagenzien nicht.

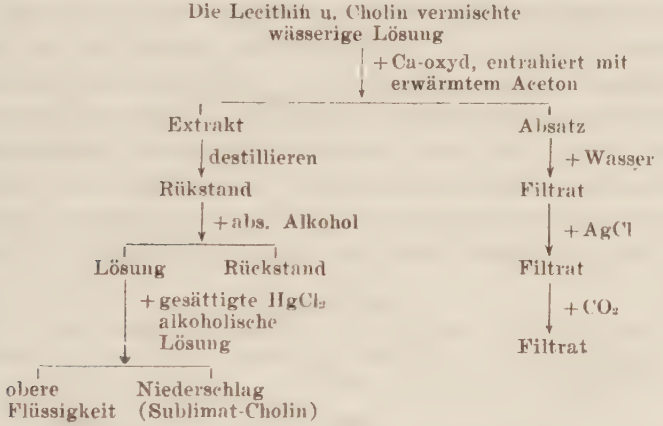
Da, wie oben beschrieben, durch Beimischung von Calciumoxyd zu altem Lecithin und durch Extraktion mit kochendem Aceton in der Flüssigkeit Colamin und im Satz des Acetonextraktes Cholin enthalten ist, goss man zum Satz Wasser hinzu, rührte und filtrierte. Dies wurde dreimal widerholt, auch der Salz auf dem Filtrierpapier wurde dreimal gewaschen, dann das Filtrat mit dem Waschwasser zusammengetan, woraus sich eine etwas dickliche Wasserlösung von 50 ccm ergab. Dieser mischte man Silberoxyd bei, filtrierte sie, wusch dreimal, mengte das Filtrat mit dem Waschwasser, führte ferner Kohlenoxyd zu und filtrierte die erhaltene Fällung. Diese wusch man dreimal, und die ganze Flüssigkeit liess man im Vakuum verdampfen und konzentrieren, entnahm eine kleine Menge davon, der man das gleiche Quantum Picrinsäurelösung beifügte, und liess das Ganze in der Eiskammer erkalten. Am nächsten Tag konnte man zwischen dem Kristall der Picrinsäure ein wenig nadelförmigen Kristall bemerken. Diesen entnahm man, löste ihn in 5 ccm Wasser auf und stellte ihn in die Eiskammer. Als man ihn am nächsten Tag makroskopisch besah, konnte man doch keinen nadelförmigen Kristall des Cholin-Picrats finden; jedoch mikroskopisch gesehen, waren zwischen dem Kristall der Picrinsäure einige nadelförnige Kristalle zu entdecken.

Ausserdem nahm man 5 ccm von der konzentrierten Flüssigkeit, die man im Vakuum verdampfen und trocknen liess, und der entstandenen hellgelblich-braunen sirupösen Substanz fügte man reinen Alkohol hinzu, zerrührte alles gut, filtrierte und wusch das Filtrierpapier dreimal. Dann mischte man das Filtrat mit dem Waschwasser, konzentrierte es im Wasserbad, füllte es in ein Zentrifugierglas um, wusch das alte Röhrchen dreimal und goss das Waschwasser wiederum zum Filtrat, sodass man etwa 10 ccm erhielt. Diesem mischte man 5 ccm reinalkoholisch gesättigte Sublimatlösung bei, die obere klare Flüssigkeit goss man ab und wusch die Fällung dreimal mit reinem Alkohol. Danach betrug deren Menge nur noch etwa $\frac{1}{3}$. Diese Fällung wurde verascht, mit Ammonium-Molybdat Phosphor nachgewiesen und nach

Rasseigne's Methode der Stickstoff geprüft.

Man weiss, dass man aus dem Satz des mit Calciumoxyd vermischten und durch Aceton extrahierten Lecithin das Cholin mit Wasser extrahieren kann, jedoch zur gleichen Zeit Lecithin ins Wasser übergeht.

Um zu sehen, ob diese Methode gut oder schlecht ist, haben wir mit der Mischung des Lecithins und Cholins Versuche gemacht und der Reihenfolge nach auf folgende Tabelle gebracht:



Als Bestimmungsmaterial haben wir folgende zwei Arten gebraucht:

a) eine Mischung von 2 ccm 0,1% wässerige Cholin-Chlorid-Lösung und 2 ccm 1%ige wässrige Ovo-lecithin (von Merck) Lösung.

b) als Kontrolle 2 ccm 0,1% wässerige Cholin-Chlorid-Lösung, gemischt mit 2 ccm Wasser.

Die Extraktionsdauer betrug sowohl bei a) wie bei b) dreimal je 5 Min..

Von beiden bereitete man je zwei Lösungen vor, denen man je 3 g Calciumoxyd beimischte und gut zerrührte, brachte einen Kühler an und extrahierte im Wasserbad mit 30 ccm Aceton. Der Extrakt wurde filtriert, der Satz von Kolben und Filtrierpapier mit kochendem Aceton zehnmal gewaschen, das Wachaceton und das Filtrat zusammengegossen und im Vakuum konzentriert.. Dem

nicht mehr nach Aceton riechenden sirupösen Rückstand gab man 10 ccm reinen Alkohol bei und schüttelte gut, filtrierte die alkoholische Lösung, wusch den Satz auf dem Filtrierpapier mit ein wenig reinem Alkohol fünfmal ab, konzentrierte das Filtrat zusammen mit dem Waschalkohol auf 2 ccm, füllte alles in ein Zentrifugierglas, fügte diesem reinalkoholisch gesättigte Sublimatlösung bei und erhielt damit die Fällung des Cholin-Sublimats. Bei a) bildete sich bei allen Extraktionsmethoden sehr wenig gelbliche, klebrige Fällung, die an der Röhrchenwand und am Glasstäbchen haften blieb. Die Fällung bei b) war weiss und nicht klebrig und blieb auch nicht an der Röhrchenwand und am Stäbchen hängen. Nachdem die 4 Arten der a) und b) Fällungen alle erst zweimal mit reinem Alkohol, dreimal mit Äther, ferner zweimal mit reinem Alkohol gewaschen worden waren, wurde die Stickstoffmenge bestimmt (Microkjeldahl) und auch die Cholinchlorid-Menge berechnet. Das Resultat war wie Tabelle VI zeigt:

Tabelle VI.

Nr. des Experiments	Gefundene *N*-Menge (mg)	Errechnete HCl-Cholin (mg)
a { 1	0,049	0 488
a { 2	0,052	0,517
b { 1	0,056	0,558
b { 2	0,042	0,419
Theoretisch. Wert		2,000

Als nächstes gab man Cholin zu Lecithin, mischte dies mit Calciumoxyd, goss zu dem Satz nach dem Extrahieren mit Aceton Wasser zu; nach dreimaligem Waschen goss man das Waschwasser über das Filtrat, fügte dieser Mischung Silberoxyd hinzu und filtrierte. Hierauf führte man Kohlensäure zu und, nachdem man filtriert hatte, goss man das Waschwasser abermals über das Filtrat und liess alles im Vakuum konzentrieren. Zu der konzentrierten Lösung gab man die gleiche Menge alkoholische Pierinsäure-Lösung bei und liess alles in der Eiskammer erkalten. Als man an nächsten Tag nachsah, konnte man ausser dem Kristall der Pierinsäure auch ein wenig Cholinpicrat bemerken. Dieses wurde entfernt und

schnell mit kaltem Wasser gespült. Nach Rasseigne's Methode war der Stickstoff positiv. Bei dem Versuch mit Cholin allein als Kontrolle konnte man dieses Kristall in grösserer Menge nachweisen.

Ausserdem behandelte man die Mixtur von je 2 ccm 1% wässeriger Lecithinlösung und 0,1% wässeriger Cholin-Lösung auf dieselbe Weise und mischte der konzentrierten Flüssigkeit solange reinalkoholisch gesättigte Sublimat-Lösung bei, bis keine Fällung mehr möglich war, zentrifugierte und wusch die Fällung mit reinem Alkohol fünfmal. Zur Kontrolle behandelte man Cholin allein auf die gleiche Weise. Beim Bestimmungsmaterial wurde die hellgelbliche klebrige Fällung durch Wachen mit reinem Alkohol immer weniger, jedoch war das bei der Kontrolle nicht der Fall. Beide Fällungen wurden je in einen Kjeldahlkohlben umgefüllt, oxydiert und die Stickstoffmenge bestimmt (nach Mikrokjeldahl). Das Ergebnis war, wie folgende Tabelle zeigt:

Tabelle VII.

		N (mg)	HCl-Cholin (mg)
	I.	0,074	0,739
	II.	0,090	0,893
Kontroll.	I.	0,113	1,126
	II.	0,123	1,226
Theoretisch. Wert			2,000

Obige Tabelle zeigt, dass das Cholin weniger war als die erwartete Menge und dass es auch bei der Kontrolle ziemlich wenig war. Ferner, dass sich in der Aceton-Flüssigkeit noch Cholin befand, das bedeutet, dass dies nicht ganz ausgeschieden werden konnte.

Bei den oben beschriebenen Versuchen ging in die Aceton-Flüssigkeit Lecithin und Cholin über, ferner ging auch Lecithin in die durch Extrahieren mit Wasser des Aceton-Extraktsatz entstandene Flüssigkeit über, und zugleich konnte man feststellen, dass die Menge des extrahierten Cholins sehr viel weniger war als

der erwartete Wert.

Um die oberen Versuchen mehr bestimmt zu machen, brachte man je 2 ccm 0,1% reinalkoholische Cholinchlorid-Lösung in zwei Zentrifugiergläser, fügte direkt reinalkoholisch gesättigte Sublimat-Lösung hinzu und erzielte dadurch die Fällung des Cholin-Sublimat-Doppelsalzes. Die Fällungen wurden ganz genau auf die gleiche Weise wie a und b behandelt und die Stickstoffmenge bestimmt. Das Resultat zeigt folgnde Tabelle:

Tabelle VIII.

Experiment	Gefundene *N*-Menge (mg)	Errechnete HCl-Cholin (mg)
1	0,180	1.793
2	0,179	1,786
Theoretisch. Wert		2 000

Zwei Gründe möchte ich erwähnen, warum bei den bis jetzt beschriebenen Versuchen nach der Levene und Ingvaldsen-Methode die Unterschiede zwischen dem erwarteten und experimentellen Wert sehr gross sind:

1) Das Lecithin löst sich im kochenden Aceton auf.

2) Nach Roman (1930) haben Cholin und Cholinchlorid die Eigenschaft, durch Kochen bei 40° zerstört zu werden. Da das im Bestimmungsmaterial enthaltene Cholin durch Extrahieren mit kochendem Aceton gespalten wird, hat dies auf die bestimmte Cholinmenge Einfluss.

Wir gaben zu 0,5 g Lecithin 20 ccm Aceton und, nachdem dies 5 Min. im Wasserbad gekocht worden war, wurde es filtriert, wobei man ein klares dickliches Filtrat erhielt. Nun wiesen wir bei einem kleinen Teil mit Ammoniummolybdat Phosphor und nach Lasseigne's Methode Stickstoff nach. Den Rest des Filtrats konzentrierten wir im Vakuum und extrahierten den sirupösen Rückstand mit Äther, filtrierten den Ätherextrakt, entfernten den Äther und erhielten eine klebrige lecithinartige Substanz. Diese war in Aceton schwer, in Äther, Choloroform und Alkohol aber leicht löslich und enthielt Phosphor und Stickstoff. Man kann

wohl annehmen, dass die Aceton-Extrakt-Lösung der früheren (Kapitel II) und jetzigen Versuche und die durch Beimischung von reinalkoholisch gesättigter Sublimat-Lösung entstandene Fällung darum klebrig sind, weil Lecithin darin enthalten ist. Da das Extrahieren des Cholins mit kochendem Aceton also vernunftwidrig ist, kann man auch das erhoffte Resultat nicht erzielen.

IV. Nachprüfung der Roman-Methode.

Scharpe (1923) baute seine Methode auf der Meinung von Stanek (1905), auf, und Roman (1930) modifizierte diese, indem er mit Roman'schem Reagenz, d.h. mit Kalium-Trijodid Cholin in Cholin-Perjodid machte ($C_5H_{15}O_2NJ–J_8$), fällte das darin gebundene Jod mit Natrium-Thiosulfat titrierte und berechnete dadurch die Cholin-Menge.

Versuchsmethode:

Reagenzien: 1) Kaliumtrijodid-Lösung mit destilliertem Wasser auffüllen bis 1000,0 ccm
Jod 157,0 g
Kaliumjodid 200,0 g
2) Chloroform
3) *n*/100 Thiosulfatlösung
4) 1% wässerige Stärkelösung

(Die gewählten Reagenzien müssen möglichst rein sein.)

Als Bestimmungsmaterial wurde wässerige Cholin-Chlorid-Lösung gebraucht. Von dem 0,01%, 0,0025%, 0,001%, 0,0005% Bestimmungsmaterial wird je 1 ccm in ein spitzes Zentrifugierglas mit Glasstopfen gegeben, das man neutralisierte oder schwach säuerte, dem man 0,3 ccm Roman'sches Reagenz beimischte und bei 3000 Umdrehungen 30 Min. zentrifugierte. Dann dekantierte man äusserst schnell und vorsichtig die obere klare Flüssigkeit. In diesem Fall sinkt nicht alles Kristall des Cholin-Perjodids auf den Boden des Spitzglases; wenn beim Abgiessen der oberen klaren Flüssigkeit etwas mitfliessen sollte, filtriert man mit einem Asbestfilter. Dann wird die im spitzen Zentrifugierglas enthaltene Fällung und der am Absbestfilter hängende Rest viermal schnell

mit 5 ccm Wasser von nahe zu 0° gewaschen; das letzte Waschwasser muss ganz durchsichtig sein und darf keine Jodfärbung mehr haben. Das Kristall des im spitzen Zentrifugierglas enthaltenen Cholin-Perjodids wird mit 2 ccm Chloroform und das im Asbestfilter mit 3 ccm aufgelöst und beides in ein spitzes Zentrifugierglas zusammengegossen, mit $n/100$ Triosulfatlösung die Jodmenge titriert, indem man nach jedem Tropfen gut schüttelte (für diesen Zweck wählte man ein Zentrifugierglas mit Glaspfropfen). Wenn man bei dieser Behandlung nicht eiskalt wäscht und nicht sehr schnell arbeitet, löst sich das Cholin-Perjodid durch Erhöhung der Temperatur des Wassers in diesem auf und fliesst ab; darum gebraucht man, um schnell filtrieren zu können, beim Waschen des im Asbestfilter enthaltenen Restes die Wasserstrahlpumpe. Als Indikator gab man vor dem Titrieren 1 Tropfen 1% Stärkelösung bei und schüttelte gut. Wenn man die Chloroformlösung mit Stärkelösung mit der ohne diese vergleicht, so erkennt man, dass der Emulsionszustand bei ersterer länger anhält und Thiosulfat auf Jod schneller wirkt.

Berechnung: Die Berechnung beruht darauf, dass in 1 Molekül Cholin-Perjodid 1 Molekül Cholin und 9 Atome Jod enthalten sind. Die Cholin- und Jodproportion beträgt 121,128: $126{,}92 \times 9$, d.h. 13.45:126,92. 1 ccm $n/100$ Natrium-Thiosulfatlösung bindet 1 ccm $n/100$ Jodlösung (= 1,2692 mg Jod), was 0,1345 mg Cholin entspricht. 1 ccm $n/100$ Thiosulfat-Lösung = 0,1345 mg Cholin und für 1 ccm $n/100$ Thiosulfat-Lösung ist 0,15499 mg Cholinchlorid.

Da man die zu diesem Versuchsmaterial dienende wässerige Cholinchlorid-Lösung nach zwei Methoden herstellte, teilte man die Versuchsresultate auch in zwei verschiedene ein. Das gebrauchte Cholinchlorid war alles Ishizu's Präparat.

1) Das in der Wagschale befindliche Cholinchlorid wurde mittels Desikkator im Vakuum getrocknet, bis das Gewicht konstant wurde, dann goss man in die Wagschale Wasser, sodass man 0,01% erhielt. Auf gleiche Weise bereitete man 0,0025%, 0,001%, 0,0005% wässerige Cholinchlorid-Lösung, die man als Versuchsmaterial verwendete, und bestimmte auf oben erwähnte Weise die Cholinmenge.

Tabelle IX a.

0,01% HCl-Cholinlösung (ccm)	Gefundene Cholin-Menge (γ)	Als HCl-Cholin (γ)
1	85,866	98,946
1	87,831	101,210
1	87,529	100,862
1	87,075	100,340
1	86,017	99,120
Mittelwert		100,096

Das erhoffte Resultat war, Cholin 92,7884γ und Cholinchlorid 100.00γ. der gefundene Wert war jedoch 100,1%.

Tabelle IX b.

0,0025% HCl-Cholinlösung (ccm)	Gefundene Cholin-Menge (γ)	Als HCl-Cholin (γ)
1	22,071	25,433
1	21,618	24,911
1	21,831	25,126
Mittelwert		25,156

Der erhoffte Wert der Cholinchlorid war 25,0γ und der gefundene Wert 100,62%.

Tabelle IX c.

0,001% HCl-Cholinlösung (ccm)	Gefundene Cholin-Menge (γ)	Als HCl-Cholin (γ)
1	8,468	9,758
1	8,768	10,104
1	8,617	9,930
1	8,466	9,755
1	8,617	9,929
Mittelwert		9,895

Das erhoffte Resultat der Cholinchlorid war 10,0γ, der gefundene Wert 98,95%.

Tabelle IX d.

0,005% HCl-Cholinlösung (ccm)	Gefundene Cholin-Menge (γ)	Als HCl-Cholin (γ)
1	4,233	4,878
1	4,079	5,062
1	4,082	4,703
Mittelwert		4,881

Das erhoffte Resultat der Cholinchlorid war 5,0γ, der gefundene Wert 97,62%.

Wann man die 4 Tabellen betrachtet, zeigen sich verhältnismässig keine schweren Fehler, und der Mittelwert der vier Resultate war 99,3228%. Unsere Resultate stimmen ziemlich mit denen von Roman mit einem durchschnittlichen Fehler von $\pm$1,70% überein.

2) Da das Cholinchlorid Feuchtigkeit anzieht, wogen wir ca. 0,1 g Cholinchlorid ab, lösten dies in 100 ccm Wasser auf, erhielten eine ca. 0,1% Lösung und bestimmten die Stickstoff-Menge nach Mikrokjeldahl's Methode, um die wirkliche Konzentration feststellen zu können. Die Stickstoff-Menge zeigt folgende Tabelle:

Tabelle X a.

Ca. 0,1% HCl-Cholinlösung (ccm)	$n/100$ H_2SO_4 (ccm)	Benötigte $n/100$ $Na_2S_2O_3$ Lösung (F = 1,1395) (ccm)	Errechnete N-Menge (γ)
10	10	3,34	867,184
10	10	3,35	865,589
10	10	3,35	865,589
10	10	3,37	862,397
Mittelwert			865,165

Die Stickstoffmenge des gesamten Bestimmungsmaterials betrug 865,165, die der Leerversuche 45,626, daher war die wirkliche

Tabelle X b. (Leerversuch).

Destill. Wasser (ccm)	$n/100$ H_2SO_4 (ccm)	Benötigte $n/100$ $Na_2S_2O_3$ Lösung (F = 1,1395) (ccm)	Errechnete N-Menge (γ)
10	10	8,49	45,626
10	10	8,49	45,626

in dem Bestimmungsmaterial enthaltene Menge 865,165 − 45,626 = 819,539. Von diesem Resultat aus berechnet ist in 10 ccm obiger Lösung $139{,}58 \times 819{,}539/14{,}008\gamma = 8166{,}137\gamma$ Cholinchlorid enthalten; d.h. auf 1 ccm Lösung fällt $816{,}614\gamma$ Cholinchlorid. In Folgenden nehmen wir diese Zahl als theoretischen Wert.

1 ccm 0,0816614% wässerige Cholinchlorid-Lösung gebrauchte man als Bestimmungsmaterial, bestimmte nach obiger Methode das Cholin und erhielt folgendes Resultat:

Tabelle XI.

0,0816614% HCl-Cholinlösung (ccm)	Benötigte $n/100$ $Na_2S_2O_3$ Lösung (F = 1,1743) (ccm)	Errechnete HCl-Cholin Menge (γ)
1	4,46	808,769
1	4,47	813,595
1	4,48	815,408
Mittelwert		812,590
	(F = 1,0493)	
1	5,09	827,700
1	4,95	805,070
1	5,01	815,325
1	4,93	801,111
1	5,06	823,262
1	5,15	837,905
1	4,86	790,722
1	4,81	782,587
Mittelwert		810,460

Der Tabelle nach war das Resultat 99,38%, des theoretischen Wertes und der durchschnittlichem Fehler ±0,62%. Es zeigt auch keine verhältnismässig schweren Fehler, sie stimmen mit denen von Roman ziemlich überein.

Die Resultate der Nachprüfung der Roman'schen Bestimmungsmethode des Cholins waren, wie oben gezeigt, verhältnismässig gut, doch konnten wir bemerken, dass, wenn man bei der Behandlung nicht äusserst vorsichtig und schnell arbeitet, sich schwere Fehler in den Resultaten einstellen.

3) Bestimmung der Cholinmenge in der mit Lecithin gemischten Cholinlösung.

Da wir von Anfang an den Zweck verfolgt haben, die Cholinmenge der Mischung von Cholin und Lecithin zu bestimmen, haben wir geprüft, welchen Wert die Roman'sche Methode bei der mit Lecithin gemischten Cholinlösung hat.

Man msichte daher als Versuchsmaterial 0,5 ccm 1% wässerige Lecithin-Lösung mit 0,5 ccm 0,005% Cholinchlorid-Lösung, und behandelte sie auf obige Weise. Das gebrauchte Lecithin war Ovo-Lecithin von Merck, welches sehr unrein ist, wie schon früher erwähnt. Bei diesem Versuch gab man dem Bestimmungsmaterial 1 ccm Roman'sches Reagenz bei; nach dem Zentrifugieren fand man auf dem Boden des Spitzglases ausser der Fällung des Cholin-Perjodids eine gelblich-braune Fällung, die, wenn man sie mit dem Glasstäbchen zerrührte, nicht wie Cholin-Perjodid brüchig war und wie eine klebrige Salbe an dem Glasstäbchen hängen blieb. Als man mit Wasser von 0° wusch, blieb nicht nur die Fällung des Cholin-Perjodids, sondern auch die andere zurück und wurde sie, wie auch der dunkelgrüne Cholin-Perjodid-Kristall, mit jeder Waschung weniger. Nach dem Waschen und Zentrifugieren war die obere Flüssigkeit nicht ganz klar; nach dem Zerrühren der gelblichbraunen Substanz, weiterem Waschen und Zentrifugieren wurde eine gänzliche Trübung hervorgerufen. Hier ist auch wie bei der Cholin-Lösung allein die Menge der Fällung nach 3–4 Waschungen nicht bestimmt, manchmal nimmt sie ab, manchmal wird sie kaum weniger. Nach viermaligem Waschen mit Wasser von 0° wurde sie verascht und Phosphor festgestellt.

Tabelle XII.

Gemischte Lösung des Lecithins u. HCl-Cholins (ccm)	Gefundene N-Menge (γ)	Errechnete HCl-Cholin (γ)
1	22,11	25,478
1	17,42	20,074
1	14,07	16,213
1	12,43	14,319
1	19,16	22,081
1	24,79	28,566
1	11,39	13,125
1	8,84	10,191
Mittelwert		18,756

Die Fällung nach viermaligem Waschen wurde in Chloroform aufgelöst eine 1% Stärke-Lösung als Indikator beigegeben, mit Thiosulfatlösung titriert und die Cholinmenge berechnet.

Wie die Tabelle zeigt, sind die Fehler gross und die Bestimmungsmethode hat als solche keinen grossen Wert. Dies kommt wohl daher, dass das Lecithin und Trijodid mit Cholin-Perjodid zusammen fällt und dass beim Waschen die beiden ersten letztere schleppen und zusammen zu einer kolloiden Lösung werden, und dass dies auf die Jodmenge der Fällung grossen Einfluss ausübt.

Um Cholin-Perjodid aus Lecithin und Jod sich ausscheiden zu lassen, kann man ausser destilliertem Wasser von 1° kein anderes Lösungsmittel gebrauchen, und zwar darum, weil nach Roman's Versuchen sich Jod und Cholin-Perjodid, beides in den organischen Lösungsmitteln, wie Chloroform, Äther und Aceton ganz natürlich auflöst, wie auch im Litiumchlorid, in 6% wässeriger Kochsalz-Lösung, in 33% wässeriger Magnesiumchlorid-Lösung und in 1% Natrium-Jodid. Daher ist es gut, vor dem Beimischen des Roman'schen Reagenz aus der Cholinlösung das Lecithin zu entfernen. Hierüber werden wir in folgendem Kapital berichten.

V. Über die Scheidung des beigemischten Lecithin beim Bestimmen der Cholinmenge.

Aus den obigen Versuchen wurde uns klar, dass ein Scheiden

der Fällung des Cholins vom beigemischten Lecithin unmöglich ist, wenn man zur Mischung von Cholin und Lecithin die Fällungsmittel des Cholins zugibt. Wir haben nun gefühlt, dass es wichtig ist, vor dem Bemischen der Reagenzien das Lecithin zu scheiden; wir gebrauchten als Lecithin Merck's Präparat, als Cholin das Ishizu'sche Präparat Cholinchlorid, als Bestimmungsmethode die aus den vorhergehenden Versuchen als gut befundene Roman'sche Methode und haben folgende verschiedenen Versuche ausgeführt.

1) Versuche mit Aceton.

Mischungen von je 1% Lecithin und Cholinchlorid wurde 4, 8, 16, 30 und 60 mal Aceton beigemischt, die man eine Stunde stehen liess, filtrierte, das Filtrat konzentrierte, veraschte. In der Asche konnte man mit Ammoniumolybdat positiv Phosphor nachweisen. Daraus ersieht man, dass das Lecithin nicht vollkommen gefällt, sondern im Filtrat mit Cholin zusammen aufgelöst worden war.

Dem Versuchsmaterial wurde 3, 5, 7 und 10 mal Magnesiumchlorid gesättigte oder Magnesiumsulfat gesättigte Aceton-Lösung beigemischt, die obere Flüssigkeit verascht und in der Asche Phosphor nachgewiesen.

2) Versuche mit Salz.

Hierauf versuchten wir mit verschiedenen Salzen allein, das Lecithin zu fällen. Zuerst versuchten wir das mit Lecithin allein. Man fügte von einem der Fällungsmittel, nämlich Magnesiumchlorid, Calciumchlorid, Eisenhydroxyd und Eisencitrat, 1 ccm dem Lecithin hinzu und, um den Phosphor in der oberen Flüssigkeit nachzuweisen, veraschte man 1 Stunde nach dem Beimischen des Fällungsmittels die obere Flüssigkeit, und führte mit Ammoniummolybdat den Nachweis.

Was man an dieser Tabelle besonders beachten muss, ist das Rusultat der 5–10% Eisencitrat-Lösung. Man kann aber annehmen, dass ein vollständiges Ausfällen des Lecithins durch Salz nicht unmöglich ist.

Um bei den bisherigen Versuchen zu prüfen, ob die Reagenzien im Vergleich zum Lecithin genügen, haben wir von den ver-

Tabelle XIII.

Fällungsmittel		Niederschlag	Obere Flüssigkeit	Phosphor in oberer Flüssigkeit
$MgCl_2 \cdot 6H_2O$	*n*/16	einwenig	Klar	+
	n/32	„	„	++
	n/64	Spur	schwach getrübt	+++
$CaCl_2 \cdot 6H_2O$	*n*/32	einwenig	Klar	+
	n/64	„	„	++
	n/128	Spur	stark getrübt	+++
$Fe(OH)_3$	1%	rötlich braun u. Spur	schwach getrübt	+++
	3%	weisslich braun u. Spur	„	+++
	5%	gelblich weiss einwenig	„	+++
Eisen citrat	1%	Spur	einwenig getrübt	++
	2%	einwenig	Klar	+
	3%	„	„	+
	5%	„	„	±
	10%	viel	„	±

schiedenen wässerigen Salzlösungen mit verschiedenen Konzentrationen die am besten die Fällung hervorrufenden, nämlich *n*/16 $MgCl_2 \cdot 6H_2O$, *n*/32 $CaCl_2 \cdot 6H_2O$, 10% Ferrum citricum und 1% $Fe(OH)_3$ gewählt, 1 ccm dieser wässerigen Salzlösung zu 1 ccm 1% wässeriger Lecithin-Lösung beigegeben und letztere ausfällen lassen, in die obere Flüssigkeit hat man einen Tropfen einer wässerigen Salzlösung eingelassen; doch entstand nirgends eine Fällung, nämlich wir wissen, dass die beigemischte Salzmenge genügend war.

3) *Versuche mit Salzmixturen.*

Aus je 1 ccm der vier oben erwähnten Lösungen mit ver-

schiedenen Konzentrationen, nämlich $MgCl_2 \cdot 6H_2O$, $CaCl_2 \cdot 6H_2O$, $Fe(OH)_3$ und Ferrum citricum wurden verschiedene Mischungen hergestellt und die gemischte Lösung zu 2 ccm wässeriger Lecithin-Lösung gegeben. Ausser dem Gebrauch dieser 2 ccm sind die Bedingungen die gleichen wie bei den vorigen Versuchen. Die Resultate erscheinen auf der folgenden Tabelle.

Tabelle XIV.

$n/16$ $MgCl_2 \cdot 6H_2O$ 1 ccm + Eisencitratlösung von verschied. Konz. 1 ccm.

Konzentration d. Citrat	Niederschlag	Obere Flüssigkeit	Phosphor in oberer Flüssigkeit
1%	Spur	klar	++
2%	„	„	+
3%	einwenig	„	+
5%	„	„	±
10%	viel	„	±

Wenn man dieses Resultat näher betrachtet, erkennt man, dass die Ausfällungswirkung je nach der Steigerung der Konzentration der mit $n/16$ $MgCl_2 \cdot 6H_2O$-Lösung gemischten Ferrum citricum-Lösung sich verstärkt.

Tabelle XV.

$n/32$ $CaCl_2 \cdot 6H_2O$ 1 ccm + Eisencitratlösung von verschieden Konz. 1 ccm.

Konzentration d. Eisencitrat	Niederschlag	Obere Flüssigkeit	Phosphor in oberer Flüssigkeit
1%	Spur	klar	++
2%	„	„	+
3%	einwenig	„	+
5%	„	„	±
10%	„	„	±

Wie die Tabelle zeigt, wird die Fällung des Lecithins je nach der Steigerung der Konzentration Ferrumcitricum-Lösung der gemischten Lösung vermehrt.

Tabelle XVI.

n/32 $CaCl_2 \cdot 6H_2O$ 1 ccm + verschieden konz. $Fe(OH)_3$ 1 ccm.

Konzentration d. $Fe(OH)_3$	Niederschlag	Obere Flüssigkeit	Phosphor in oberer Flüssigkeit
1%	rot, schwammig	klar	+
5%	rötlich braun	„	+
10%	braun	„	+

Tabelle XVII.

n/16 $MgCl_2 \cdot 6H_2O$ 1 ccm + verschieden konzentrierte Eisenhydroxyd 1 ccm.

Konzentration d. $Fe(OH)_3$	Niederschlag	Obere Flüssigkeit	Phosphor in ober. Flüssigkeit
1%	rot, schwammig	klar	+
5%	rötlich braun	„	+
10%	braun	„	+

Bei diesen beiden Versuchen konnte man kein gutes Fällungsmittel finden.

Tabelle XVIII.

10% Eisencitrat 1 ccm + verschied. konzentrierte Eisenhydroxyd 1 ccm.

Konzentration d. $Fe(OH)_3$	Niederschlag	Obere Flüssigkeit	Phosphor in oberer Flüssigkeit
1%	gelblich weiss	geblich klar	±
5%	gelblich braun	gelblich braun klar	±
10%	braun	„ „ „	+

Bei diesen Versuchen konnte man erkennen, dass durch Beimischung von 1–5% $Fe(OH)_3$ zu 10% Ferrum citricum das Lecithin fast vollkommen gefällt wurde.

Tabelle XIX.

n/16 $MgCl_2 \cdot 6H_2O$ 1 ccm + verschieden konz. $CaCl_2 \cdot 6H_2O$ 1 ccm.

Konzentration d. $CaCl_2 \cdot 6H_2O$ u. d. $MgCl_2 \cdot 6H_2O$	Niederschlag	Obere Flüssigkeit	Phospor in oberer Flüssigkeit
Jedes *n*/16	einwenig	klar	+
Jedes *n*/32	„	„	+
Jedes *n*/64	Spur	schwach getrübt	+ +
Jedes *n*/128	„	stark getrübt	+ + +

Nach den obigen Tabellen sind die Resultate gleich denen des Ausfällens durch $MgCl_2 \cdot 6H_2O$ oder $CaCl_2 \cdot 6H_2O$ gesonderte Lösung mit verschiedenen Konzentrationen.

In der folgenden Tabelle wollen wir diejenigen verschiedenen Salzlösungen zeigen, die eine vollkommene Fällung des Lecithins hervorzurufen imstande sind. Zu 2 ccm dieser verschiedenen Lösungen gab man die gleiche Menge oder fünfmal soviel 1% wässerige Lecithin-Lösung und prüfte in der oberen Flüssigkeit den Nachweis des Phosphors und Stickstoffs.

Tabelle XX.

Lecithinlösung	Ausfällungsmittel		In oberer Flüssigkeit	
			Phosphor	Stickstoff
1% Lecithin-Lösung 1 ccm (=0,01 g)	10% Eisencitrat $n/16$ $MgCl_2 \cdot 6H_2O$	0,5 ccm 0,5 ccm	–	–
„	10% Eisencitrat $n/32$ $CaCl_2 \cdot 6H_2O$	0,5 ccm 0,5 ccm	–	–
„	10% Eisencitrat	10 ccm	–	–
„	10% Eisencitrat 5% $Fe(OH)_3$	0,5 ccm 0,5 ccm	–	–
1% Lecithin-Lösung 10 ccm (=0,1 g)	10% Eisencitrat $n/16$ $MgCl_2 \cdot 6H_2O$	5 ccm 5 ccm	+	+
„	10% Eisencitrat $n/32$ $CaCl_2 \cdot 6H_2O$	5 ccm 5 ccm	+	+
„	10% Eisencitrat	10 ccm	–	–
„	10% Eisencitrat 5% $Fe(OH)_3$	5 ccm 5 ccm	–	–

Aus dieser Tabelle ersieht man, dass durch Beimischung von 10% Ferrum citricum-Lösung allein oder einer Mischung mit dieser und $n/16$ $MgCl_2 \cdot 6H_2O$ oder $n/32$ $CaCl_2 \cdot 6H_2O$ oder 5% $Fe(OH)_3$ zu der kleinen Menge (nämlich 1 ccm) von 1% Lecithin eine Ausfällung erfolgreich ist. Doch bei einer grossen Menge (nämlich 10 ccm) 1% Lecithin ist dies nicht der Fall, durch Beimischen von 10% Ferrum citricum allein oder einer Mischung von diesem und 5% $Fe(OH)_3$ entstand eine Fällung; jedoch kam bei der Mischung mit $n/8$ $MgCl_2 \cdot 6H_2O$ keine vollkommene Fällung zustande. Es ist

klar, dass im Gegensatz dazu die beim Ausfällen des Lecithins durch Ferrum citricum allein hervorgerufene Fällung schnell und vollkommen war, bei einer Mischung von Eisencitrat und Magnesiumchlorid oder Calciumchlorid ausser einer Fällung des Lecithins durch Ferrum citricum auch eine gewisse durch diese Salze entsteht und beide Fällungen vermischt werden; es ist schon erwähnt, dass ein vollständiges Ausfällen des Lecithins durch Magnesium-oder Calcium-Salze nicht möglich ist, Das Resultat des Ausfällens war beim Gebrauch von Ferrum citricum allein und auch bei Mischung von diesem und $Fe(OH)_3$ gleich, und die Bedeutung des Vorhandenseins von $Fe(OH)_3$ in der Mischung mit Ferrum citricum wird darin gefunden, dass die hervorgerufene Fällung durch $Fe(OH)_3$ adsorbiert wird, also daher das Verfahren der Scheidung aus der Lecithin-Cholin-Mischung erleichtert wird und das Waschen und Filtrieren leicht ist, dagegen der durch Mischung von Ferrum citricum und $MgCl_2 \cdot 6H_2O$ oder $CaCl_2 \cdot 6H_2O$ hervorgerufene Niederschlag leicht ist und sich zerstreut.

Diesen obigen Resultaten nach ist das Fällen des Lecithins durch 10% Eisencitratlösung oder die Mischung von 5% Eisenhydroxyd und 5% Eisencitrat am erfolgreichsten.

VI. Bestimmungsmethode des Cholins in der gemischten Lecithin-Cholin-Lösung nach dem Ausscheiden des Lecithin.

Nach den obigen Versuchen geschieht die Bestimmungsmethode des Cholins in der gemischten Lecithin-Cholin-Lösung nach dem Ausscheiden des Lecithins wie folgt:

Als Bestimmungmaterial gebrauchten wir die Mischung von 1 ccm 1% Lecithin (Merck's Präparat) und 1 ccm 0,0816614 Cholinchlorid (Ishizu's Präparat). Von dieser Mischung gab man 2 ccm in ein spitzes Zentrifugierglas und als Lecithin-Ausfällungsmittel fügte man zuerst ca. 1 ccm 10% Eisencitrat-Lösung bei und erhielt schnell und deutlich eine gelbliche Lecithin-Fällung. Diese wurde zwei oder-dreimal gekippt und gemischt, und nachdem sie 10 Min. lang mit 3000 Umdrehungen zentrifugiert war, wurde

1 ccm der zweiten Ausfällungslösung, einer Mischung gleicher Menge von 5% wässeriger Eisencitrat und 5% wässeriger Eisenhydroxyd-Lösung beigemischt und noch einmal zentrifugiert. Das durch die erste Lösung ausgefällte und an der Spitzglaswand hängende Lecithin sank auf den Boden und wurde zugleich durch das Eisenhydroxyd zu Boden gehalten, daher schwamm es bei dem nächsten Filtrieren nicht oben auf; also war das Entfernen der oberen Flüssigkeit durch Filtrierpapier sehr leicht. Das Filtrat wurde durch feines Filtrierpapier in ein anderes Spitzglas gebracht. Das Filtrierpapier wurde solange mit destilliertem Wasser gewaschen, bis das Gelb des Ferrum citricum sich verlor (dabei ist es sehr bequem, dass das Ferrum citricum gelb ist; weil es ein gutes Merkmal ist und zeigt, ob das Filtrat gut gewaschen wurde oder nicht). Das Filtrat und die Waschflüssigkeit wurden im Spitzglas gesammelt und das Cholin in dieser Flüssigkeit nach der früher erwähnten Roman'schen Methode bestimmt.

Tabelle XXI.

Benötigte $n/100$ $Na_2S_2O_3$ Lösung (F = 1,1743) (ccm)	Errechnete HCl-Cholin Menge (γ)
4,46	812,148
4,43	805,948
4,44	807,498
4,43	805,948
(F = 1,0493)	
4,94	802,848
4,93	801,298
4,97	809,048
Mittelwert	806,390

Die Resultate sind, wie die obige Tabelle zeigt:

Das erhoffte Resultat war 816,614, der durchschnittliche, gefundene Wert 806,390 (98,75%), der durchschnittlich Fehler also 1,25%.

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STUDIES ON THE ALKALI HYDROLYSIS OF DIKETOPIPERAZINES AND ANILINE PEPTIDES AND ITS DISSOCIATION CONSTANTS.

(Directed by Assist. Prof. Dr. T. Uchino.)

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Introduction.

Michaelis and Menten (1913) explained the process of enzymatic hydrolysis with the assumption of the formation of easily dissociable intermediary compounds of enzyme and substrate. In the formation of such intermediary compounds some specific chemical affinity between enzyme and substrate seems to play a part.

Northrop (1922) examined the action of pepsin and trypsin on several proteins and showed that pepsin is active only on positively charged protein cations, trypsin on negatively charged anions, and that the union of enzyme and substrate is largely determined by electrical forces of both substances. According to the same author (1924) trypsin behaves as a positively charged cation in the solution of pH 2.0 to 10.2 and the enzyme-substrate union is determined by the electronegative character of substrate.

Ishiyama (1933) has recently shown that diketopiperazines containing free carboxyl group such as glycyl-aspartic acid anhydride, glycyl-glutamic acid anhydride, and aspartyl-aspartic acid anhydride are all hydrolyzed by carboxypolypeptidase, whereas none of non-carboxylated diketopiperazines are attacked at all. The presence of carboxyl group in the substrate-molecule seems to determine the enzymatic cleavability of diketopiperazines.

Balls and Köhler (1931) investigated the action of erepsin

on a number of aniline peptides, i.e. peptides composed of glycine and aniline derivatives and found that erepsin can hydrolyze peptide without any free carboxyl group such as glycyl-*p*-nitroaniline and that of the three isomers of aminobenzoic acid peptides glycyl-*m*- and glycyl-*p*-aminobenzoic acids are readily hydrolyzed by erepsin, whereas the ortho-body can not be attacked at all. Inasmuch as glycyl-aniline can give rise to a series of derivatives with different chemical groups in the aromatic nucleus, the aniline peptides seem to be quite convenient substrates for studying the mechanism of enzymatic hydrolysis. The above authors pointed out that the union of peptide and enzyme occurs at two points. The first point, where the substrate molecule is united with enzyme, is the free amino group and the second linking point the imino group of the peptide linkage. This second linkage is said to induce the hydrolytic cleavage and therefore a certain acid character of the imino group is said to determine the enzymatic cleavability of the substrate.

It seems therefore interesting for the author to examine and compare some physico-chemical properties of several diketopiperazines and aniline peptides. In the present series of experiments we compared on the one hand the rates of alkali hydrolysis of these substances and on the other hand, the electrometric titration was performed in order to determine the acidic and basic dissociation constants of these substances and to examine the state of dissociation at varying pH of the solution, inasmuch as the electrical charge of the substrate seems to play an important part in the formation of enzyme-substrate compounds.

Preparation of diketopiperazines.

Glycyl-glycine anhydride. This substance was prepared from glycine ethyl ester by the method of Fischer and Fourneau (1901). The crystals purified by recrystallization from water, showed no biuret reaction. The substance, dried at 110°, gave N, 24.27%. Calculated for $C_4H_6N_2O_2$, N, 24.56%.

Glycyl-alanine anhydride and *Glycyl-tyrosine anhydride.* The products, obtained by Assistant Prof. Uchino in the course of his

studies on the partial hydrolysis of silk-fibroin (T. Uchino, 1934) were used in this experiment. The Glycyl-alanine anhydride melted at 238° and the glycyl-tyrosine anhydride at 272°. The former gave, N, 21.71% (calculated for $C_5H_8N_2O_2$, N, 21.87%) and the latter, N. 12.67% (calculated for $C_{11}H_{12}N_2O_3$, N. 12.72%).

Glycyl-aspartic acid anhydride, *glycyl-glutamic acid anhydride* and *aspartyl-aspartic acid anhydride* were all prepared according to the method described by Ishiyama (1933). The glycyl-aspartic acid anhydride melted at 217° and gave, N, 16.17% (calculated for $C_6H_8N_2O_4$, N, 16.28%). The glycyl-glutamic acid anhydride melted at 225° and gave, N, 15.05% (calculated for $C_7H_{10}N_2O_4$, N, 15.05%). The aspartyl-aspartic acid anhydride showed no definite melting point and gave, N, 11.94% (calculated for $C_8H_{10}N_2O_6$, N, 12.18%).

Preparation of aniline peptides.

Glycyl-aniline. Monochloracetanilide was prepared from chloracetyl-chloride and aniline by the method of Meyer(1875). Glycyl-aniline was then prepared from monochloracetanilide thus obtained by the method of W. Majert (1918). The peptide was isolated as hydrochloride: M. P. 218°. The substance, dried at 110°, gave, N, 14.80%. Calculated for $C_8H_{10}N_2O.HCl$, N, 15.02%.

Glycyl-o-toluidine. The hydrochloride was prepared in exactly the same manner as in the case of glycyl-aniline. The substance melted at 246° and gave, N. 13.79% (calculated for $C_9H_{12}N_2O.HCl$, N. 13.97).

Glycyl-p-Chloraniline. The substance was prepared by the method of Balls and Köhler (1931). It melted at 75° and gave, N. 14.83% (calculated for $C_8H_9N_2OCl$, N. 15.18%).

Glycyl-o-aminobenzoic acid. Chloracetyl-*o*-aminobenzoic acid was prepared from *o*-aminobenzoic acid as described by Waldschmidt-Leitz and Balls (1931). This substance was then allowed to stand with 30 per cent ammonia at 37° for 12 hours and the solution was concentrated under reduced pressure. The precipitates, which separated out, were dissolved in water by the carefull addition of dilute ammonia and reprecipitated by acidifica-

tion with hydrochloric acid. For further purification it was recrystallized from hot water: M. P. 235–236°. The substance gave, N, 14.13% (calculated for $C_9H_{10}N_2O_3$, N, 14.43%).

Glycyl-m-aminobenzoic acid. This was prepared from *m*-aminobenzoic acid by the method of Tropp (1928). The recrystallized substance melted at 224°C and gave, N, 14.09% (calculated for $C_9H_{10}N_2O_3$, N, 14.43%).

Glycyl-p-aminobenzoic acid. The Substance was prepared in a same manner as that for glycyl-*m*-aminobenzoic acid. It melted at 227.5° and gave, N, 14.37% (calculated for $C_9H_{10}N_2O_3$, N, 14.43%).

Comparison of the rates of alkali hydrolysis of diketopiperazines.

Levene and his co-workers (Levene, Bass and Steiger, 1929; Levene, Rothen, Steiger and Osaki, 1930) have studied, in some detail, the rates of alkali hydrolysis of various diketopiperazines, but the compounds with free carboxyl group were not yet examined by them. The experimental procedure in this paper is almost the same as described by these authors.

The hydrolysis of diketopiperazines was carried out at 25° and in 0.5 *N* NaOH, 5 mols of NaOH per mol of diketopiperazine being used. In the case of diketopiperazines of acid nature, consuming 1 or 2 equivalents of alkali, the additional NaOH necessary for neutralization was employed. A sample of 0.0025 mol was weighed in a 25 cc. volumetric flask and dissolved in a little distilled water. 12.5 cc. (5 equivalents) of 1.0 *N* NaOH were then added and the volume was made up to 25 cc. In the case of glycyl-tyrosine anhydride, glycyl-aspartic acid anhydride and glycyl-glutamic acid anhydride 15.0 cc. (6 equivalents) and in the case of aspartyl-aspartic acid anhydride 17.5 cc. (7 equivalents) of 1.0 *N* NaOH were added, since these substances combine with one or two equivalents of alkali. The solution was mixed as rapidly as possible and kept in a thermostat adjusted at 25°. 3.0 cc. samples were withdrawn from the solution at definite time intervals and emptied into a series of conical flasks containing 3.0 cc. of 0.5 *N*

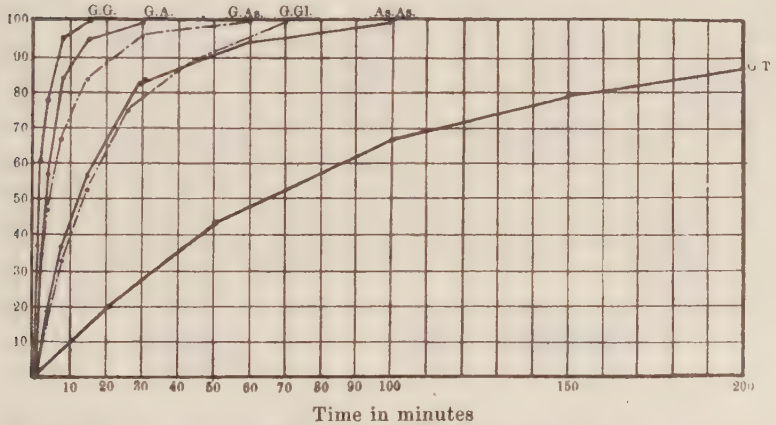
HCl. The amino nitrogen content of the solution thus neutralized was then determined by Linderström-Langs method (1928).

In this experiment the assumption was made that no further hydrolysis of the peptides into free amino acids took place. This secondary hydrolysis occurs under the present experimental conditions so slowly that the error due to this seems to be quite insignificant. The results obtained are shown in Table I and Fig. 1. The values of k were calculated by means of the equation of unimolecular reaction:

$$k = \frac{1}{t} \log \frac{a}{a-x} = \frac{1}{t} \{\log a - \log(a-x)\}$$

t being the time in minutes.

Fig. 1. Alkali hydrolysis of diketopiperazines in 0.5 N NaOH at 25°.



G. G. Glycyl-glycine anhydride
G. A. Glycyl-alanine anhydride
G. As. Glycyl-aspartic acid anhydride
G. Gl. Glycyl-glutamic acid anhydride
As. As. Aspartyl-aspartic acid anhydride
G. T. Glycyl-tyrosine anhydride

The results show that the reaction constants of glycyl-glycine anhydride, glycyl-alanine anhydride and glycyl-tyrosine anhydride agree fairly well with those reported by Levene and his coworkers. Glycyl-glycine anhydride and glycyl-alanine anhydride are most rapidly hydrolyzed. Glycyl-aspartic acid anhydride is

TABLE I. Rates of hydrolysis of diketopiperazines in 0.5 *N* NaOH at 25°.

Glycyl-glycine anhydride				Glycyl-alanine anhydride				Glycyl-tyrosine anhydride			
Time in minutes	*N*/10 HCl cc.	Hydrolysis %	K.10^3	Time in minutes	*N*/10 HCl cc.	Hydrolysis %	K.10^3	Time in minutes	*N*/10 HCl cc.	Hydrolysis %	K.10^3
1	0.92	30.67	159.00	2	1.05	35.00	93.55	10	0.21	7.00	(3.15)
2	1.51	50.33	151.95	4	1.72	57.33	92.48	20	0.59	19.67	4.76
4	2.33	77.67	162.75	8	2.53	84.33	100.63	50	1.27	42.33	4.78
8	2.84	94.67	159.13	15	2.85	95.00	(86.73)	100	1.98	66.00	4.69
15	2.97	99.00	(133.33)	30	3.01			150	2.36	78.67	4.47
30	3.02			60	3.04			200	2.60	86.67	4.38
60	3.05										
Average			158.21				95.55				4.62

Glycyl-aspartic acid anhydride				Glycyl-glutamic acid anhydride				Aspartyl-aspartic acid anhydride			
Time in minutes	*N*/10 HCl cc.	Hydrolysis %	K.10^3	Time in minutes	*N*/10 HCl cc.	Hydrolysis %	K.10^3	Time in minutes	*N*/10 HCl cc.	Hydrolysis %	K.10^3
2	0.85	28.33	(72.35)	2	0.20	6.67	(14.95)	4	0.55	18.33	21.98
4	1.38	46.00	66.90	4	0.46	15.33	18.08	8	1.10	36.67	24.79
8	1.99	66.33	59.10	8	0.97	32.33	21.20	15	1.52	50.67	20.45
15	2.50	83.33	51.87	15	1.55	51.67	21.05	30	2.48	82.67	25.37
30	2.87	95.67	(45.44)	26	2.25	75.00	23.15	60	2.83	94.33	20.78
60	2.97			44	2.67	89.00	21.79	100	2.97	99.00	20.00
100	3.01			70	2.98	99.33	(31.09)				
Average			59.29				21.05				22.23

Values of K.10^3 in parentheses were omitted in calculating average values.

hydrolyzed at a lower rate than glycyl-alanine anhydride, but more rapidly than glycyl-glutamic acid anhydride. The hydrolysis rate of aspartyl-aspartic acid anhydride is nearly the same as that for the hydrolysis of glycyl-glutamic acid anhydride, while glycyl-tyrosine anhydride is hydrolyzed at a much lower rate.

Comparison of the rates of alkali hydrolysis of aniline peptides.

The hydrolysis of aniline peptides was carried out at 35° and in 1*N* NaOH, 10 mols of NaOH per mols of peptide being used. A quantity equivalent to 0.0025 mol of aniline peptides was weighed in a 25 cc. volumetric flask and dissolved in 12.5 cc. of 2*N* NaOH and the volume was made up to 25 cc. with distilled water. The amount of NaOH added was thus 10 equivalents. The solution was mixed as rapidly as possible and placed in a thermostat adjusted at 35°. From time to time 3.0 cc. samples were withdrawn from the solution and emptied into a series of conical flasks containing 3.0 cc. 1 *N* HCl for the purpose of neutralizing alkali. To the solution were added 1 cc. 0.5 per cent alcoholic solution of thymolphthalein and 85 cc. 96 per cent alcohol and the extent of hydrolysis was titrated by the method of Willstätter and Waldschmidt-Leitz (1921), using thymolphthalein as indicator. The rate of hydrolysis was calculated by means of the equation of unimolecular reaction:

$$k = \frac{1}{t} \log \frac{a}{a-x} = \frac{1}{t} \left\{ \log a - \log(a-x) \right\}$$

t being the time in minutes. The results are shown in Table II and Fig. 2.

The results in Table II and Fig. 2 show that glycyl-*p*-aminobenzoic acid is hydrolyzed at a faster rate than glycyl-*m*-amino- and *o*-aminobenzoic acids. The hydrolysis rate of glycyl-aniline is almost the same as the latter two peptides. Glycyl-*o*-toluidine was hydrolyzed at a remarkably lower rate than the other aniline peptides.

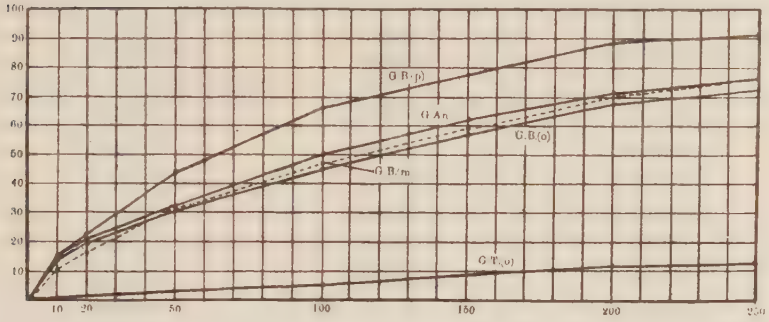
TABLE II. Rates of hydrolysis of aniline peptides in 1 *N* NaOH at 35°.

Glycyl-aniline hydrochloride				Glycyl-*o*-toluidine hydrochloride				Glycyl-*o*-amino-benzoic acid			
Time in minutes	*N*/10 KOH cc.	Hydrolysis %	K.10³	Time in minutes	*N*/10 KOH cc.	Hydrolysis %	K.10³	Time in minutes	*N*/10 KOH cc.	Hydrolysis %	K.10³
10	0.44	14.67	(6.89)	10	0	0		10	0.41	13.67	(6.38)
20	0.64	21.33	(5.21)	20	0	0		20	0.58	19.33	(4.67)
50	0.95	31.67	(3.31)	50	0.05	1.67	(0.15)	50	0.89	29.67	(3.06)
100	1.48	49.33	2.95	100	0.12	4.00	(0.18)	100	1.32	44.00	2.52
150	1.86	62.00	2.80	200	0.35	11.67	0.27	150	1.70	56.67	2.42
200	2.15	71.67	2.74	250	0.37	12.33	0.23	200	2.04	68.00	2.47
250	2.29	76.33	(2.50)					250	2.20	73.33	2.30
Average			5.83				0.25				2.43

Glycyl-*m*-amino-benzoic acid				Glycyl-*p*-amino-benzoic acid			
Time in minutes	*N*/10 KOH cc.	Hydrolysis %	K.10³	Time in minutes	*N*/10 KOH cc.	Hydrolysis %	K.10³
10	0.31	10.33	(4.73)	10	0.44	14.67	(6.89)
20	0.46	15.33	(3.62)	20	0.65	21.67	(5.30)
50	0.94	31.33	(3.26)	50	1.21	40.33	4.48
100	1.38	46.00	2.68	100	1.98	66.00	4.69
150	1.74	58.00	2.51	150	2.33	77.67	4.34
202	2.13	71.00	2.66	200	2.65	88.33	4.67
250	2.30	76.67	2.53	250	2.75	91.67	4.32
Average			2.60				4.50

Values of K.10³ in parentheses were omitted in calculating average values.

Fig. 2. Alkali hydrolysis of aniline peptides in 1.0 *N* NaOH at 35°.



Time in minutes

G. B. (*o*)	Glycyl-*o*-aminobenzoic acid	G. An.	Glycyl-aniline
G. B. (*m*)	Glycyl-*m*-aminobenzoic acid	G. T. (*o*)	Glycyl-o-toludine.
G. B. (*p*)	Glycyl-*p*-aminobenzoic acid		

Electrometric titration of diketopiperazines.

The titration curves of glycyl-glycine anhydride, glycyl-alanine anhydride and glycyl-tyrosine anhydride were determined in 0.01 mol solution as follows. To a series of test tubes containing 5.0 cc. of 0.02 mol diketopiperazine solution were added an increasing amount of *N*/50 HCl or NaOH and the contents of the tubes were diluted with distilled water so as to make the total volume of 10 cc. The pH of the resultant solutions were then determined by use of hydrogen electrode. The results are shown in Table III–V and Fig. 3–5. As glycyl-tyrosine anhydride was not readily soluble in water, it was dissolved with the addition of an equivalent amount of NaOH solution. This alkali was substrated from the amount of HCl or added to that of NaOH in the calculation.

For the titration of other diketopiperazines the electrode vessel and microburette as described by Rasmussen and Linderström-Lang (1934) were employed. 0.02 mol diketopiperazine solution was introduced into the electrode vessel and titrated with *N*/2 NaOH or HCl at 25°. The results are shown in Table VI–VIII, and Fig. 6–8.

It will be seen from Fig. 3–8 that glycyl-glycine anhydride and

Table III.
Electrometric titration of glycyl-glycine anhydride.

Glycyl-glycine anhydride molar	HCl molar	pH
0.0100	0.0100	2.000
0.0100	0.0060	2.199
0.0100	0.0040	2.388
0.0100	0.0020	2.602
0.0100	0.0010	2.867
0.0100	0.0005	3.197
0.0100	0	6.130
Glycyl-glycine anhydride molar	**NaOH molar**	**pH**
0.0100	0.0005	10.210
0.0100	0.0010	10.617
0.0100	0.0020	11.031
0.0100	0.0040	11.441
0.0100	0.0060	11.546
0.0100	0.0100	11.798
0.0100	0.01616	12.001

Fig. 3. Titration curves of glycyl-glycine anhydride.

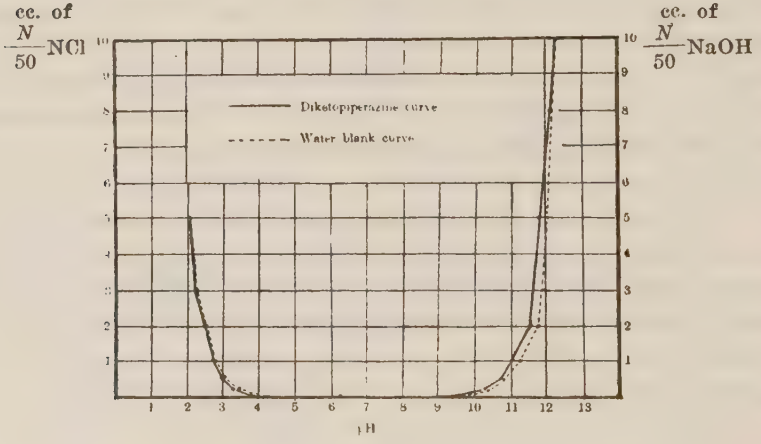


TABLE IV.

Electrometric titration of glycyl-alanine anhydride.

Glycyl-alanine anhydride molar	HCl molar	pH
0.0100	0.0100	2.022
0.0100	0.0060	2.237
0.0100	0.0040	2.424
0.0100	0.0020	2.714
0.0100	0.0010	3.007
0.0100	0.0005	3.351
0.0100	0	5.941
Glycyl-alanine anhydride molar	**NaOH molar**	**pH**
0.0100	0.0005	10.391
0.0100	0.0010	10.579
0.0100	0.0020	11.014
0.0100	0.0040	11.556
0.0100	0.0060	11.614
0.0100	0.0100	11.846
0.0100	0.01616	12.135

Fig. 4. Titration curves of glycyl-alanine anhydride.

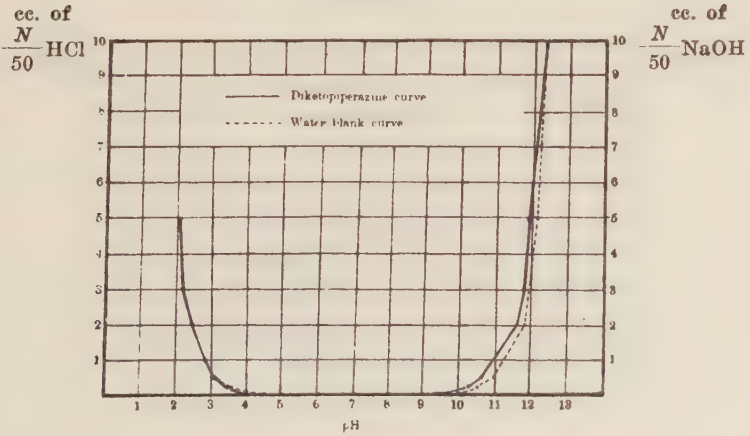


Table V.

Electrometric titration of glycyl-tyrosine anhydride.

Glycyl-tyrosine anhydride molar	HCl molar	pH	pKa
0.0100	0.0100	1.934	
0.0100	0.0060	2.066	
0.0100	0.0040	2.291	
0.0100	0.0020	2.644	
0.0100	0.0010	2.867	
0.0100	0.0005	3.306	
0.0100	0	5.207	
Glycyl-tyrosine anhydride molar	**NaOH molar**	**pH**	**pKa**
0.0100	0.0005	7.399	(8.678)
0.0100	0.0010	8.330	9.285
0.0100	0.0020	8.858	9.462
0.0100	0.0040	9.396	9.576
0.0100	0.0060	9.739	9.572
0.0100	0.0080	10.037	9.463
0.0100	0.0100	10.607	——
0.0100	0.0120	11.216	Average
0.0100	0.0140	11.591	9.472
0.0100	0.0160	11.766	
0.0100	0.0200	12.090	

Fig. 5. Titration curves of glycyl-tyrosine anhydride.

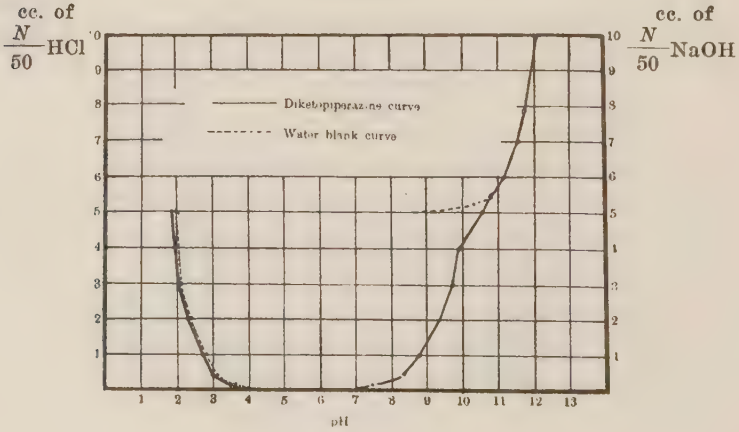


TABLE VI.

Electrometric titration of glycyl-aspartic acid anhydride (25°)

Titration with HCl			Titration with NaOH			
Glycyl-aspartic acid anhydride 0.02 mol	HCl 0.5 mol	pH	Glycyl-aspartic acid anhydride 0.02 mol	NaOH 0.5 mol	pH	pKa
cc.	cc.		cc.	cc.		
5.0	0.00	2.807	5.0	0.02	3.129	3.927
5.0	0.02	2.563	5.0	0.04	3.389	3.937
5.0	0.04	2.342	5.0	0.08	3.761	3.921
5.0	0.08	2.106	5.0	0.12	4.124	3.941
5.0	0.12	1.932	5.0	0.16	4.645	(4.040)
5.0	0.16	1.831	5.0	0.18	5.223	(4.270)
5.0	0.20	1.746	5.0	0.19	8.317	——
5.0	0.30	1.588	5.0	0.20	10.581	Average
5.0	0.40	1.496	5.0	0.22	11.120	3.931
			5.0	0.24	11.442	
			5.0	0.33	11.877	
			5.0	0.40	12.046	

Fig. 6. Titration curves of glycyl-aspartic acid anhydride.
Temp. = 25°.

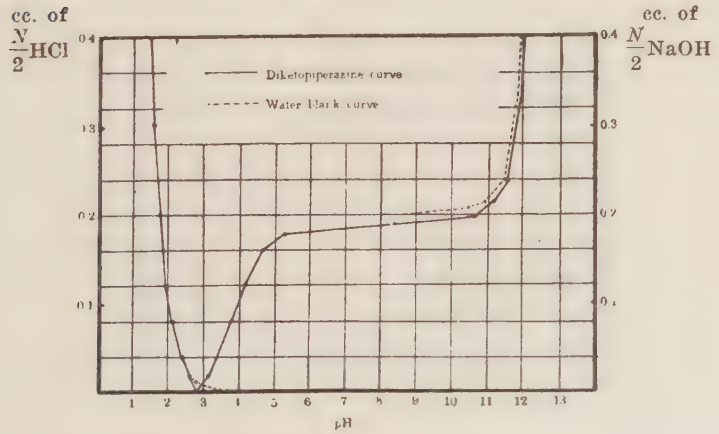


Table VII.
Electrometric titration of glycyl-glutamic acid anhydride (25°)

Titration with HCl			Titration with NaOH			
Glycyl-glutamic acid anhydride 0.02 mol	HCl 0.5 mol	pH	Glycyl-glutamic acid anhydride 0.02 mol	NaOH 0.5 mol	pH	pKa
cc.	cc.		cc.	cc.		
5.0	0.00	3.006	5.0	0.02	3.296	(4.139)
5.0	0.02	2.457	5.0	0.041	3.799	4.367
5.0	0.04	2.364	5.0	0.08	4.190	4.360
5.0	0.08	2.090	5.0	0.12	4.552	4.373
5.0	0.12	1.942	5.0	0.16	4.949	4.346
5.0	0.16	1.810	5.0	0.17	5.118	4.365
5.0	0.20	1.726	5.0	0.18	5.321	4.368
5.0	0.30	1.583	5.0	0.19	5.728	(4.451)
5.0	0.40	1.474	5.0	0.20	8.839	——
			5.0	0.22	11.039	Average 4.363
			5.0	0.24	11.244	
			5.0	0.28	11.636	
			5.0	0.40	11.751	

Fig. 7. Titration curves of glycyl-glutamic acid anhydride.
Temp. = 25°.

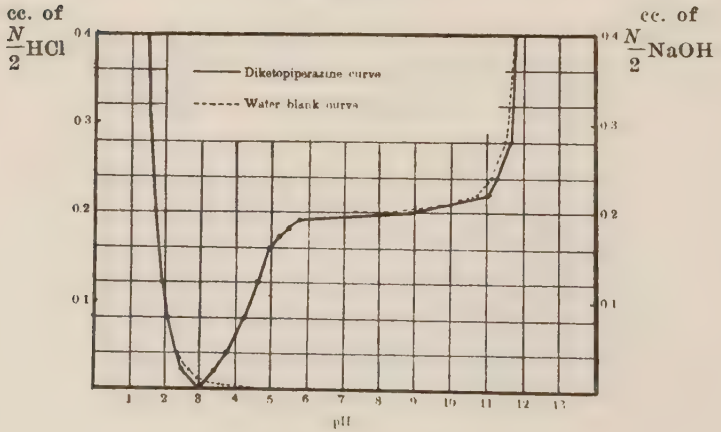
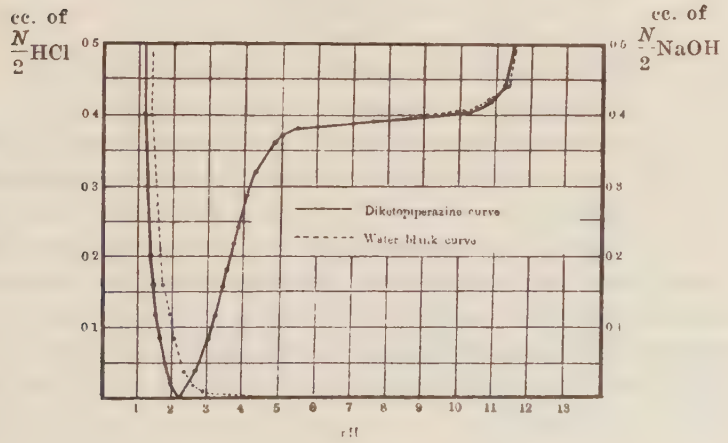


TABLE VIII.

Electrometric titration of aspartyl-aspartic acid anhydride (25°)

Titration with HCl			Titration with NaOH		
Aspartyl-aspartic acid anhydride 0.02 mol	HCl 0.5 mol	pH	Aspartyl-aspartic acid anhydride 0.02 mol	NaOH 0.5 mol	pH
cc.	cc.		cc.	cc.	
5.0	0.00	2.196	5.0	0.02	2.410
5.0	0.02	1.941	5.0	0.04	2.609
5.0	0.04	1.847	5.0	0.08	2.948
5.0	0.08	1.695	5.0	0.12	3.192
5.0	0.12	1.538	5.0	0.16	3.393
5.0	0.16	1.471	5.0	0.18	3.531
5.0	0.20	1.369	5.0	0.22	3.672
5.0	0.30	1.266	5.0	0.24	3.796
5.0	0.40	1.171	5.0	0.28	4.019
5.0	0.44	1.142	5.0	0.32	4.269
5.0	0.48	1.106	5.0	0.36	4.778
			5.0	0.37	4.975
			5.0	0.38	5.374
			5.0	0.39	7.516
			5.0	0.402	10.190
			5.0	0.42	10.880
			5.0	0.44	11.212

Fig. 8. Titration curves of aspartyl-aspartic acid anhydride.
Temp. = 25°.



glycyl-alanine anhydride show no acid- and base-combining capacity. Glycyl-tyrosine anhydride, glycyl-aspartic acid anhydride and glycyl-glutamic acid anhydride each consume one equivalent of NaOH, while aspartyl-aspartic acid anhydride behaves as dibasic acid.

It must be further noted from the titration curves that glycyl-aspartic acid anhydride, glycyl-glutamic acid anhydride and aspartyl-aspartic acid anhydride are all completely dissociated into ions at pH greater than about 6, while glycyl-tyrosine anhydride is in a ionic state at pH greater than 10.

The dissociation constants of the monobasic acids were calculated from the following equation.

$$pKa = pH + \log \frac{[S] - [B^+] - [H^+] + [OH^-]}{[B^+] + [H^+] - [OH^-]}$$

where, Ka: the dissociation constant,
[S]: concentration of total acid (diketopiperazine) and
$[B^+]$: concentration of the cation of the salt, respectively, in mols per liter.

The first and second dissociation constants of aspartyl-aspartic acid anhydride were calculated by use of the following equations:

$$\frac{[B^+]_1 + [H^+]_1}{[S]} = \frac{K_1(2K_2 + [H^+]_1)}{K_1K_2 + K_1[H^+]_1 + [H^+]_1^2}$$

$$\frac{[B^+]_2 + [H^+]_2}{[S]} = \frac{K_1(2K_2 + [H^+]_2)}{K_1K_2 + K_1[H^+]_2 + [H^+]_2^2}$$

where, K_1: the first acid dissociation constant,
K_2: the second acid dissociation constant,
[S]: concentration of total acid (diketopiperazine) and
$[B^+]$: concentration of the cation of the salt, respectively, in mols per liter.

In the above equations the assumption was made, for purposes of simplicity, that the salts of diketopiperazines are completely dissociated into ions, and that the concentration of all the constituents of the system are equal to their activities.

The results are recorded in Table V–VII and IX and summarized in Table X.

TABLE IX.

Calculation of dissociation constants K_1 and K_2 of aspartyl-aspartic acid anhydride.

Aspartyl-aspartic acid anhydride 0.02 mol	NaOH 0.5 mol	pH	pK_1	pK_2
cc.				
5.0	0.08	2.948	3.0904	4.1750
	0.18	3.531		
5.0	0.08	2.948	3.1216	3.9942
	0.22	3.672		
5.0	0.08	2.948	3.1149	4.0291
	0.28	4.019		
5.0	0.08	2.948	3.1179	4.0133
	0.32	4.269		

Average, $pK_1 = 3.111$
$pK_2 = 4.053$

TABLE X.

Dissociation constants of the diketopiperazines.

	pKa	pK_1	pK_2
Glycyl-tyrosine anhydride	9.472		
Glycyl-aspartic acid anhydride	3.931		
Glycyl-glutamic acid anhydride	4.363		
Aspartyl-aspartic acid anhydride		3.111	4.053

The value for glycyl-tyrosine anhydride was measured at 18–19°, those for other substances at 25°.

ELECTROMETRIC TITRATIONS OF ANILINE PEPTIDES.

The electrometric titrations of aniline peptides were carried out by use of the electrode vessel as described by Rasmussen and Linderström-Lang (1934). 0.02 mol solutions of aniline peptide were titrated with *N* 2 NaOH or *N* /2 HCl. As the neutral peptides were not readily soluble (glycyl-aniline and glycyl-*o*-toluidine excepted) they were dissolved in water with the addition of exactly an equivalent amount of NaOH and titrated back with HCl. As glycyl-aniline and glycyl-*o*-toluidine contain an equivalent amount of HCl as hydrochloride, they were also titrated back with NaOH. The results are shown in Table XI–XVI and Fig. 9–14.

Table XI.
Electrometric titration of glycyl-aniline (25°).

Titration with HCl				Titration with NaOH		
Glycyl-aniline 0.02 mol	HCl 0.5 mol	pH	pKb	Glycyl-aniline 0.02 mol	NaOH 0.5 mol	pH
cc.	cc.			cc.	cc.	
5.0	0.00	9.041		5.0	0.02	9.673
5.0	0.02	8.592	6.256	5.0	0.04	10.650
5.0	0.04	8.298	6.199	5.0	0.08	11.328
5.0	0.08	7.890	6.181	5.0	0.12	11.662
5.0	0.12	7.386	6.333	5.0	0.20	11.828
5.0	0.16	6.958	6.336			
5.0	0.18	6.594	6.349			
5.0	0.19	6.284	6.331			
5.0	0.20	4.499	——			
5.0	0.21	2.321	Average 6.284			
5.0	0.22	2.189				
5.0	0.24	1.841				
5.0	0.30	1.399				

Fig. 9. Titration curves of glycyl-aniline.
Temp. = 25°.

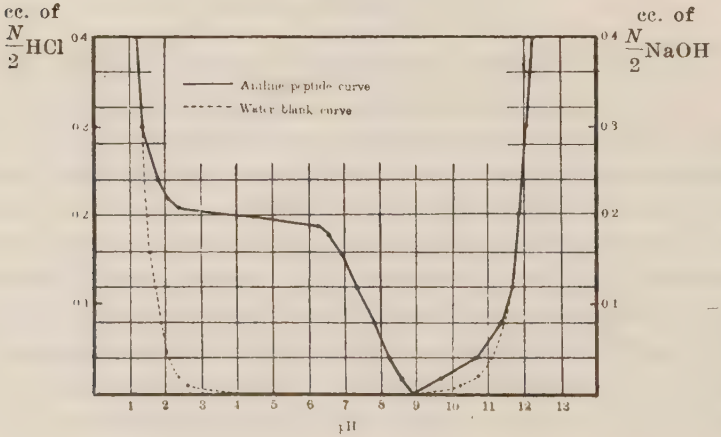


TABLE XII.

Electrometric titration of glycyl-*o*-toluidine (25°).

Titration with HCl				Titration with NaOH		
Glycyl-*o*-toluidine 0.02 mol	HCl 0.5 mol	pH	pKb	Glycyl-*o*-toluidine 0.02 mol	NaOH 0.5 mol	pH
cc.	cc.			cc.	cc.	
5.0	0.00	10.296		5.0	0.02	11.102
5.0	0.02	8.626	6.222	5.0	0.04	11.354
5.0	0.04	8.308	6.189	5.0	0.08	11.640
5.0	0.08	7.994	6.077	5.0	0.121	11.783
5.0	0.12	7.491	6.228	5.0	0.20	11.959
5.0	0.16	7.085	6.209	5.0	0.25	12.060
5.0	0.18	6.706	6.237			
5.0	0.188	6.543	6.158			
5.0	0.20	5.020	—			
5.0	0.21	2.624	Average 6.189			
5.0	0.22	2.284				
5.0	0.24	1.996				
5.0	0.30	1.660				
5.0	0.40	1.399				

Fig. 10. Titration curves of glycyl-*o*-toluidine.
Temp. = 25°.

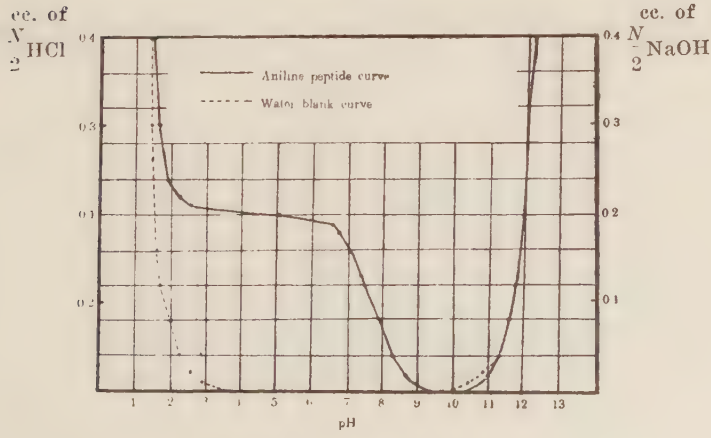


Table XIII.
Electrometric titration of glycyl-*p*-chloraniline (25°).

Titration with HCl				Titration with NaOH		
Glycyl-*p*-chlor aniline 0.02 mol	HCl 0.5 mol	pH	pKb	Glycyl-*p*-chlor aniline 0.02 mol	NaOH 0.5 mol	pH
cc.	cc.			cc.	cc.	
5.0	0.00	9.054		5.0	0.02	10.817
5.0	0.02	8.467	(6.381)	5.0	0.04	11.213
5.0	0.04	8.198	6.299	5.0	0.08	11.570
5.0	0.08	7.800	6.271	5.0	0.12	11.651
5.0	0.12	7.442	6.277	5.0	0.16	11.714
5.0	0.16	7.000	6.294	5.0	0.21	11.952
5.0	0.18	6.579	(6.364)	5.0	0.24	12.022
5.0	0.19	6.149	(6.472)			

Average, pKb = 6.285.

Fig. 11. Titration curves of glycyl-*p*-chlor-aniline.
Temp. = 25°.

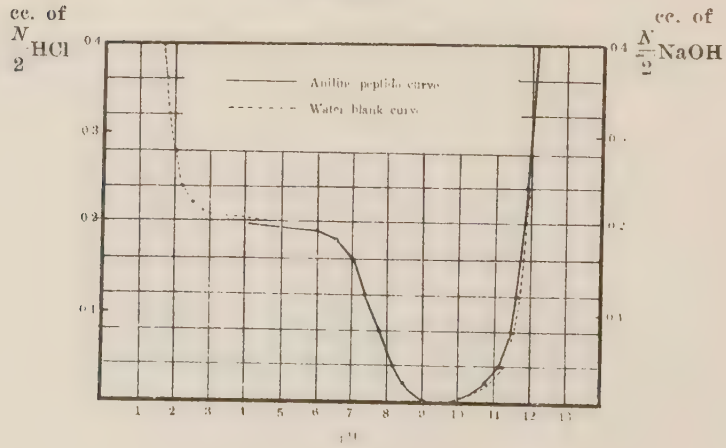


TABLE XIV.

Electrometric titration of glycyl-*o*-aminobenzoic acid (25°).

Titration with HCl				Titration with NaOH			
Glycyl-*o*-amino-benzoic acid 0.02 mol	HCl 0.5 mol	pH	pKa	Glycyl-*o*-amino-benzoic acid 0.02 mol	NaOH 0.5 mol	pH	pKb
cc.	cc.			cc.	cc.		
5.0	0.00	4.947		5.0	0.02	6.775	6.165
5.0	0.02	3.856	2.867	5.0	0.04	7.186	6.107
5.0	0.04	3.525	2.881	5.0	0.08	7.623	6.096
5.0	0.08	3.113	2.829	5.0	0.12	7.990	6.081
5.0	0.12	2.814	2.852	5.0	0.16	8.492	6.005
5.0	0.16	2.508	2.757	5.0	0.18	8.805	6.044
5.0	0.20	2.249	——	5.0	0.19	9.149	6.029
5.0	0.22	2.228	Average 2.837	5.0	0.20	10.127	——
5.0	0.24	2.071		5.0	0.22	10.986	Average 6.075
5.0	0.28	1.905		5.0	0.24	11.333	
				5.0	0.28	11.628	
				5.0	0.32	11.780	
				5.0	0.40	11.998	

Fig. 12. Titration curves of glycyl-*o*-amino-benzoic acid. Temp. = 25°.

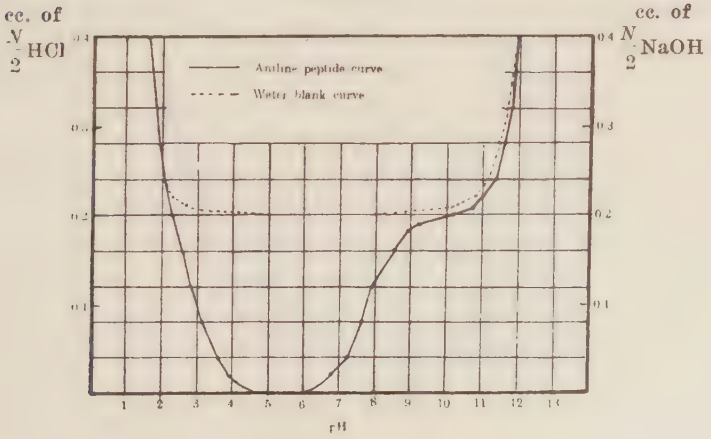


Table XV.

Electrometric titration of glycyl-*m*-aminobenzoic acid (25°).

Titration with HCl				Titration with NaOH			
Glycyl-*m*-amino-benzoic acid 0.02 mol	HCl 0.5 mol	pH	pKa	Glycyl-*m*-amino-benzoic acid 0.02 mol	NaOH 0.5 mol	pH	pKb
cc.	cc.			cc.	cc.		
5.0	0.00	5.193		5.0	0.02	6.771	(6.170)
5.0	0.02	4.509	3.537	5.0	0.031	7.105	6.054
5.0	0.039	4.120	3.493	5.0	0.08	7.762	5.957
5.0	0.08	3.729	3.536	5.0	0.10	7.920	5.975
5.0	0.12	3.357	3.493	5.0	0.16	8.464	6.032
5.0	0.164	2.942	(3.446)	5.0	0.17	8.796	(5.851)
5.0	0.202	2.518	——	5.0	0.19	9.316	(5.844)
5.0	0.242	2.195	Average 3.515	5.0	0.20	10.008	——
5.0	0.284	1.982		5.0	0.22	11.069	Average 5.952
5.0	0.40	1.682		5.0	0.24	11.391	
				5.0	0.28	11.684	
				5.0	0.40	12.022	

Fig. 13. Titration curves of glycyl-*m*-amino-benzoic acid. Temp. = 25°.

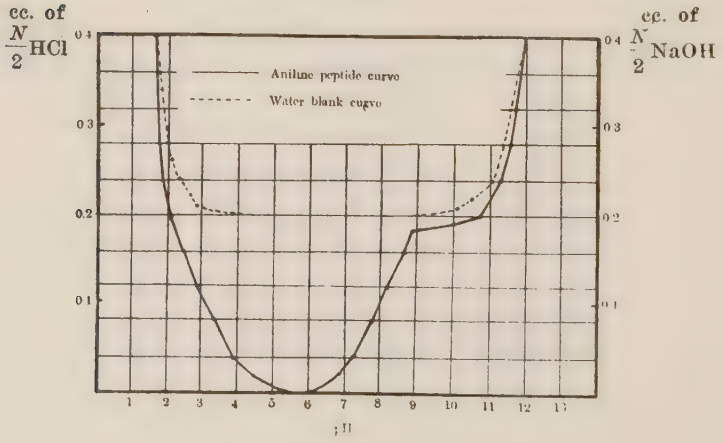
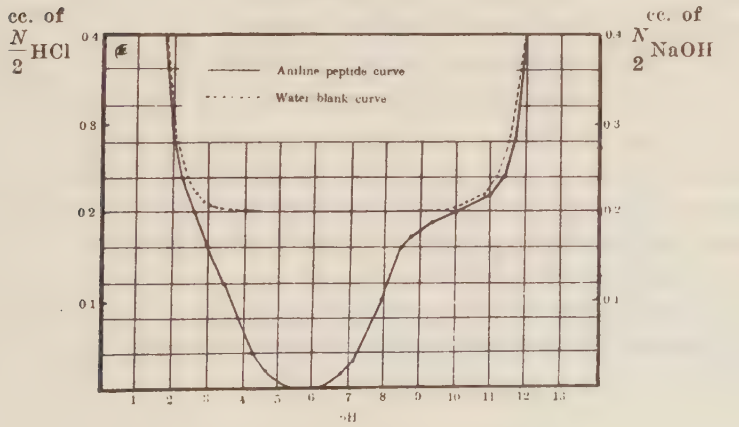


TABLE XVI.

Electrometric titration of glycyl-*p*-aminobenzoic acid (25°).

Titration with HCl				Titration with NaOH			
Glycyl-*p*-amino-benzoic acid 0.02 mol	HCl 0.5 mol	pH	pKa	Glycyl-*p*-amino-benzoic acid 0.02 mol	0.5 mol NaOH	pH	pKb
cc.	cc.			cc.	cc.		
5.0	0.00	5.390		5.0	0.02	6.792	(6.149)
5.0	0.02	4.392	(3.428)	5.0	0.04	7.215	6.078
5.0	0.04	3.863	3.242	5.0	0.08	7.728	5.991
5.0	0.08	3.433	3.223	5.0	0.119	8.136	5.926
5.0	0.12	2.865	(2.918)	5.0	0.16	8.643	5.853
5.0	0.162	2.430	(2.640)	5.0	0.18	8.870	6.002
5.0	0.20	2.042		5.0	0.19	10.077	(5.027)
5.0	0.243	1.851	Average 3.233	5.0	0.20	10.826	—
5.0	0.28	1.766		5.0	0.24	11.364	Average 5.970
5.0	0.40	1.628		5.0	0.28	11.651	
				5.0	0.322	11.795	

Fig. 14. Titration curves of glycyl-*p*-amino-benzoic acid. Temp. = 25°.



The basic dissociation constants of glycyl-aniline and glycyl-*o*-toluidine were calculated by use of the following equation.

$$pKb = pOH + \log \frac{[S] - [A^-] + [H^+] - [OH^-]}{[A^-] - [H^+] + [OH^-]}$$

where, Kb: the basic dissociation constant,
[S]: concentration of total base (aniline peptide) and
$[A^-]$: concentration of the anion of the salt, respectively, in mols per liter.

The acidic and basic dissociation constants of the other ampholytic aniline peptides were calculated according to the newer concept of ampholyte dissociation, by means of the following equations.

$$pKa = pH - \log \frac{[C] - [A] + [H^+] - [OH^-]}{[A] - [H^+] + [OH^-]}$$

where, Ka: the acidic dissociation constant,
[C]: total concentration of ampholyte and
[A]: total concentration of acid, respectively, in mols per liter.

$$pKb = pOH - \log \frac{[C] - [B] - [H^+] + [OH^-]}{[B] + [H^+] - [OH^-]}$$

where, Kb: the basic dissociation constant,
[C]: total concentration of ampholyte and
[B]: total concentration of base, respectively, in mols per liter.

In the above equations the assumptions were made, for purposes of simplicity, that the salts are completely dissociated into ions, and that the concentration of all the constituents of the system are equal to their activities.

Table XVII gives the summarized results of these calculations of dissociation constants. In the fourth column the values for the isoelectric point as given.

Table XVII.
Dissociation constants of aniline peptides (25°).

	pKa	pKb	$pI = 1/2(pKa + pKw - pKb)$
Glycyl-aniline		6.284	
Glycyl-*o*-toluidine		6.189	
Glycyl-*p*-chloraniline		6.286	
Glycyl-*o*-aminobenzoic acid	2.837	6.075	5.329
Glycyl-*m*-aminobenzoic acid	3.515	5.952	5.729
Glycyl-*p*-aminobenzoic acid	3.233	5.970	5.579

Discussion and Summary.

The rates of hydrolysis of diketopiperazines by means of alkali show us that there are no particular relations between the behaviour of diketopiperazines to alkali hydrolysis and its cleavability with enzyme. The same is also true in the case of aniline peptides and the rates of hydrolysis are not closely related with the enzymatic cleavability.

From the results of the electrometric titrations it is clear that in the vicinity of pH 8.0 all the diketopiperazines which are hydrolyzed by carboxypolypeptidase exist in a state of negatively charged anions, while other anhydrides are not dissociated into ions at this pH. This fact indicates to us that at the optimum pH of the enzyme action the negative charge of the substrate molecule may facilitate the formation of enzyme-substrate compounds as pointed out by Northrop and consequently determine the cleavability of diketopiperazines by means of enzyme. In the case of aniline peptides the titration curves show no peculiar characteristics with each other. The basic dissociation constants of all peptides are nearly identical and the acidic dissociation constants of the three aminobenzoic acid peptides are also almost the same. In the case of aniline peptides the specific properties of the substrate which cause the chemical affinities between enzyme and substrate must be further investigated from some other points of view.

In conclusion I wish to express my thanks to Professor K.

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FISHES AS THE TEST SUBJECT FOR BIOLOGICAL ACTION OF VARIOUS CHEMICAL SUBSTANCES AND THE STUDIES ON THE QUESTION ON THE CHEMICAL NATURE UREASE.

By

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I. Introduction.

In the study of biological action of many substances it is usual to inject them into vein, subcutan or intraperitoneal. Each method has however its shortage. In intravenous injection it is apt to be overdosed and besides, many substances are soon excreted through kidney, and reaction often rapidly subsides. In the subcutaneous injection the same inconveniences occur, and, beside, the resorption is not very constant. In intraperitoneal injection the reactionsmodus is not the same as in other injections.

Fish afford a good test subject because it can absorb continuously many substances through its gills from the tank water of large quantity at any required concentration. Furthermore it can only absorb crystalloidal substances. And we can distinguish whether substance in question, which calls forth reaction, is a diffusible substance or colloidal ones.

Urease is said to be of proteic nature by Sumner, while it is assumed by Waldschmidt-Leitz that the active substance is only present absorbed by protein. Sumner treated his urease with trypsin and found the activity lost, while Waldschmidt-Leitz insisted that after the trypsin treatment urease activity still existed. It is, however, also possible that the urease activity is really concerned with some chemical structure of polypeptid nature existing in the protein molecule, and if that structure is

maintained the activity is retained, even when the protein moleculer is partially split and its colloidal structure is lost.

The aim of my investigation is to find out how far fishes can be used as the test subject for biological action of various chemical substances, and also, if possible, to solve the question concerning the chemical nature of urease.

In the following, the results of experiments will be given, in which various reagents as saponine, adrenalin, atropin and insulin are given through the gills, and also the results of the efforts for clearing up the chemical nature of urease, from the modus of reaction of fish towards the differently treated urease.

II. Material and Method.

Carps (*Cyprinus carpio* L.) weighing 200–400 gm. and of 17–25 cm. of body length, were used as experimental animal. They were brought to the laboratory from the fish pond at Tamagawa and were kept in a water tank under running water at a constant temperature. In April, being the breeding period of that fish, and in February and August, owing to the extremely cold or warm water temperature, the experiment was not conducted.

For the experiment the fish which had been kept in water at 20° for a while was put into a glas water tank containing 15 liters of water at 20° and after half an hour the material to be tested was put into the tank. To examine the blood the fish was taken out when needed, fixed by the method of M. Henze (1927), and the blood was taken by the heart puncture. The injection syringe was inserted into Bulbus arteriosus, which lies about 8 mm. deep under the middle of the line connecting the frontal margins of bilateral pectoral fins, to the direction of ventriculus. The coagulation of the blood was avoided by the addition of potassium oxalate or sodium citrate in an amount of 0.02–0.03 per cent. As in summer time the blood is apt to be hemolysed, regardless of the care taken, the experiment was interrupted in August.

To avoid the action of test material from the digestive tract a paraffin block was inserted to the pharynx at the beginning of the experiment. This precaution was, however, soon discontinued,

owing to the following facts. 1) Fish, which had been kept in 5% suspension of animal charcoal for two hours contained no trace of it in the digestive tracts. 2) Fish kept in 1 mg% solution of congored for 2.5 hours showed neither colouring in digesting tractus directly, nor after the addition of acid in various parts of digestive organs. 3) The fish was kept in 1% Lithion carmin solution for 7 hours. However the digestive tract showed no coloration. 4) H. W. Smith (1932) also states "The fresh water teleost apparently does not drink water, but absorbs it through the oral membrane or perhaps through the gills."

III. Experiment with saponin.

1 gm. of saponin was dissolved in 50 cc. of water and gently added to 5 litres of water in which the carp had been kept for half an hour. As soon as saponin was added the fish began to lose the equilibrium of the body and shook its head bilaterally. After 20 to 30 minutes the fish laid out completely flat. The clearing motion of operculum, which occurs every 15–20 seconds, appeared quite short and became indistinguishable with the respirative motion. After 30 minutes the clearing motion became quite feeble.

As is already known saponin causes hemolysis to experimental animals. This was also the case in my experiment. The number of red corpuscles of the normal carp averages to about 2,112,000 per cm. The body length of carps examined varied between 18.0–20.6 cm.

As the number of red corpuscles may vary with several conditions the same number of control animals were always used in every experiment. The effect of saponin treatment of carp on number of its erythrocytes is shown in Table 1 and Fig. 1.

As is clear from the table and figure the number of erythrocytes of carp, which is kept in saponin solution, is decreased by nearly 40 per cent in the course of 30 minutes, and this decrease is rapidly attained in the first 15 minutes. This fact shows to us that saponin is easily absorbed through the gill and causes the hemolysis. No thrombosis was observed in my experimental animals.

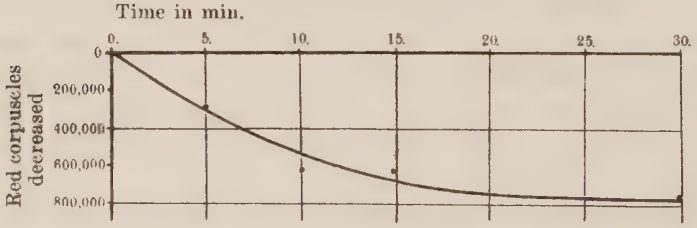
Table I.

Numbers of red blood corpuscles of carps which were kept in saponin solution.

Time	Control			Experiment			Difference	Average
	sex	body length	No. of red corpuscles	sex	body length	No. of red corpuscles		
min.		cm.			cm.			
5	♂	21.0	1,816,000	♀	23.1	1,612,000	−204,000	
„	♀	21.5	1,860,000	♀	21.9	1,540,000	−320,000	
„	♀	20.8	1,908,000	♀	21.2	1,592,000	−316,000	−280,000[3]
10	♀	21.0	1,248,000	♀	22.8	828,000	−420,000	
„	♂	21.0	1,904,000	♀	21.8	1,036,000	−868,000	−604,000[2]
15	♂	21.6	2,204,000	♂	23.0	1,492,000	−712,000	
„	♂	20.1	1,460,000	♂	22.2	968,000	−592,000	−602,000[2]
30	♂	21.8	1,998,000	♀	21.8	1,036,000	−957,000	
„	♂	22.0	1,826,000	♂	22.5	1,224,000	−602,000	
„	♀	22.5	2,112,000	♀	21.8	1,344,000	−768,000	−772,000[3]

Fig. 1.

Graphical representation of Table I.



IV. Absorption of adrenalin through the gill.

In the previous section I have shown that saponin is easily absorbed through the gill and causes the destruction of red blood cells. In this section the action of adrenalin is examined. As it is clear in land animals that adrenalin injected into animals, calls forth the hyperglycemia and the increase of creatine content of muscle tissues, these two points were examined in the following experiments.

Adreanalin was added in different doses to 5 or 10 liters of

water containing a carp, and after the lapse of certain time either the sugar content of blood or the creatine content of muscles was determined. For the determination of blood sugar the Hagedorn-Jensen (1923) method was followed, while the determination of creatine was made just as Akatsuka (1927) performed in this laboratory before several years ago.

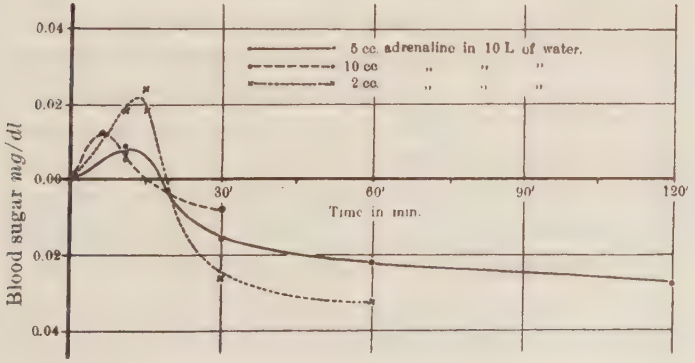
1. *The change of blood sugar content after the application of adrenalin to carp.*

The results of blood sugar determination of carp kept in adrenalin solution are given in Table II and Fig. 2.

TABLE II.
The increase or decrease of blood sugar content of the adrenalin treated fish.

Time	Amount of adrenalin added to 10 liters of water		
	2 cc.	5 cc.	10 cc.
min.	*mg/dl*	*mg/dl*	*mg/dl*
5	+0.008	−0.002	+0.012
10	+0.019	+0.010	+0.003
15	+0.022	—	—
30	−0.026	−0.016	−0.008
60	−0.032	−0.022	—
120	—	−0.029	—

Fig. 2.
The increase or decrease of blood sugar in carps treated with diff. amount of adrenalin. Water temp. 20±1°C.



Carps kept in adrenalin solution retained all normal behaviours except a retardation of the gill clearing motion, which slows down to double the normal interval. It is clear from the table and figure that the effect of adranalin acted through the gill does not differ much from that given by injection.

2, The change of creatine content of dorsal muscle of carp treated with adrenalin through the gill.

The normal amount of creatine in dorsal muscles or normal carp is averaged as 453 mg%. This value nearly coincides with that of Akatsuka (1927).

Table III.
Creatine content of dorsal muscle of normal carps at water temp. 20°C.

Time in water	Creatine	Difference
min.	mg. %	
90	476	+23
35	487	+34
45	445	−08
„	412	−41
„	430	−23
35	444	−09
210	471	+18
150	461	+08
Average	453	

In Table IV and Fig. 3 the change of creatine content after the application of adrenalin was followed by different time intervals. Each figure is the average taken from at least 3 experiments.

From the table and figure it is clear that adrenalin in an amount given as in the experiment increases the creatine content of dorsal muscle to the maximum after 60 minutes and decreases again to attain the normal value after 180 minutes.

To decide whether this return to normal value is due to the disappearence of adrenalin from the tank water or to the adapta-

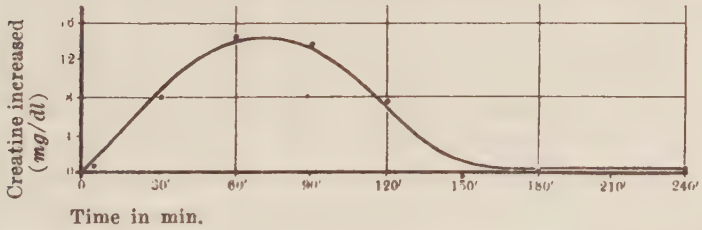
Table IV.

Change of the creatine content of dorsal muscle of carp, kept in adrenalin solution (1 cc. adrenalin added to 5 liters of water.)

Time min.	Absolute amount	Difference	%
	mg/dl		
Normal	453		
5	466	+31	+ 2.8
30	490	+37	+ 8.2
60	520	+67	+14.8
90	515	+62	+13.7
120	486	+33	+ 7.3
150	449	− 4	− 0.88
180	453	0	0
240	465	+12	+ 2.6

Fig. 3.

The effect of adrenalin on the creatine content of dorsal muscle of carps. (1 cc. adrenalin chloride in 5 *l* of water at 20°C wat. temp.)



tion of the fish, the determination of adrenalin content of the water after 3 hours is desirable. As however the sensibility of the chemical methods of adrenalin measurement is unable for this purpose, we have decided to solve this question by the same biological reaction as the experiment itself. Namely after the lapse of 3 hours, a new carp was put into the water tank instead of the previous animal and determined the amount of creatine of its dorsal muscles after 1 hour. As Table V.a and Fig. 4.a show the increase of creatine remains only to 0.66% which indicates that adrenalin is destroyed during the 3 hours.

That the return to normal value of creatine contents of muscle

is not caused by the adaptation of the animal can be shown in an experiment in which 1 cc. of adrenalin is added to the same tank after 3 hours of previous application of adrenalin. As Table V.b and Fig. 4.b indicate, 19.6% increase of creatine content is observed after one hour, showing to us the ability of the fish to react to a renewed application of adrenalin.

I have also found that 3 hours exposure of adrenalin in tank water to air and experimental temperature is not the cause of disappearence of adrenalin by an experiment, in which 1 cc. of adrenalin was added to 10 liters of tank water and a carp was put into it after the lapse of 3 hours. The amount of creatine content of muscle increased by 13.6 percent after one hour, as Table V.c and Fig. 4.c show, indicating that adrenaline remained almost unchanged in water at least for 3 hours.

Considering that the secrete or excrete from carp may play some destructive action on adrenalin, an experiment was made, in which a carp was placed in the tank water for 3 hours, afterwards the fish was removed and 1 cc. of adrenalin solution was added to it, and after the lapse of 3 hours the carp was put into the tank again. The increase of creatine content of muscle of this animal examined after one hour amounted to 11.7 percent, as is shown in Table V.d and Fig. 4.d. This result indicates to us that inactivation of adrenalin is partly caused by the secrete or excrete of the fish. The total disappearence of adrenalin during 3 hours of the main experiment is caused, therefore, only by the presence of fish itself.

To get some idea about the intensity of adrenalin destruction by the fish, two more experiments were conducted, in one of which a carp was placed for half an hour in adrenalin solution, while in the other a carp remained for one hour, both carps were taken out of the tank and a fresh carp was put into each tank. The results of determination of creatine content in both experiment are shown in Table V.e, V.f, Fig. 4.e and Fig. 4.f. We can see from them that adrenalin is almost destroyed by the presence of carp during one hour under our conditions.

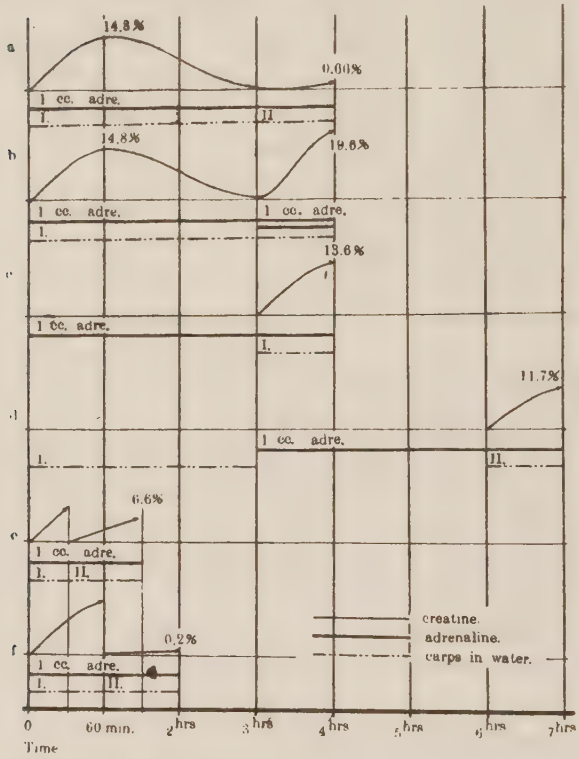
In summarizing up the above experiments, adrenalin is absorbed by the gill and causes the increase of creatine content of

TABLE V.
(at 20°C Temp.)

No.	Conditions of experiment	Creatine	Average	Stand. divi.	Difference*	%
		mg/dl	*mg/dl*		*mg/dl*	
a	Put 1 cc. adrenalin and carp, after 3 hours replace it with other carp, after 60 minutes muscle examined	465 461 445	456	±4.9	+ 3	+0.66
b	Put 1 cc. adrenalin and carp, after 3 hours another 1 cc. adrenalin added, after 60 minutes creatine determined	548 548 533	542	±4.2	+89	+19.6
c	Put 1 cc. adrenalin after 3 hours a carp added, after further 60 minutes creatine examined	533 518 500	515	±7.1	+62	+13.6
d	Put a carp, after 3 hours remove the carp and put 1 cc. adrenalin and 3 hours after other carp put into it and then after 60 minutes creatine examined	513 506 500	506	±2.6	+56	11.7
e	Put a carp and 1 cc adrenalin, after 30 minutes replaced with another carp, after further 60 minutes creatine examined	471 500 482 477	482	±6.9	+30	+6.6
f	Put 1 cc. adrenalin and a carp, after 60 minutes replace the carp with other one, after further 60 miutes creatine examined.	460 449 445 445	453	±3.4	+ 1	+0.2

* Difference from normal value.

Fig. 4.
The effect of various conditions on adrenalin destruction, examined by the amount of creatine of muscle
(Water temp. 20°C)



muscle. This is the result which Akatsuka (1927) got in this laboratory many years ago by the injection of adrenaline to the carp.

V. Application of atropin through the gill.

Akatsuka (1927) observed the fact that when atropin sulphate was injected to albino rat, in an amount of 0.2 mg per 100 gm. of its body weight, causes the decrease of creatine content of muscle. In the following the result of experiments are given in which atropin was applied through the gill to the carp.

Atropin sulphate was applied in three different concentrations of 1:5,000,000, 1:500,000 and 1:50,000. The determination of creatine content of muscle was made after definite intervals. The results are shown in Table VI and Fig. 5.

As can be seen from the table and figure, atropin applied in above concentrations always increases the creatine content of

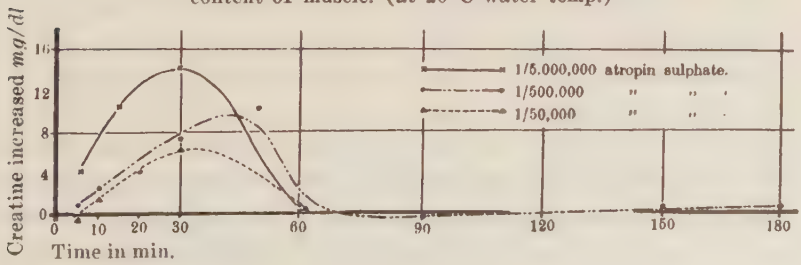
TABLE VI.

The increase or decrease of creatine content of muscle by atropin applied through the gill.

Time	Concentration of atropin applied					
	1:5,000,000		1:500,000		1:50,000	
	Absolute deff.	Percentage	Absolute deff.	Percentage	Absolute deff.	Percentage
min	*mg/dl*		*mg/dl*		*mg/dl*	
5	+18	+ 4.0	+ 2	+ 0.4	− 4	−0.9
10	+47	+10.4	+10	+ 2.2	+ 7	+1.5
15	—	—	—	—	—	—
20	—	—	+18	+ 4.0	—	—
30	+67	+14.8	+34	+ 7.5	+29	+6.4
45	+ 1	+ 0.1	+48	+10.6	—	+0.2
60	—	—	+ 1	+ 0.2	+ 1	—
90	—	—	− 8	− 1.8	—	—
150	—	—	+ 2	+ 0.4	—	—
180	—	—	+ 2	+ 0.4	—	—

Fig. 5.

The effect of atropin of various concentration on the creatine content of muscle. (at 20°C water temp.)



muscle. Parallel with this increase the fish seemed to become excited.

This finding differs from that obtained by Akatsuka, in whose experiment atropin was applied by injection and resulted in the decrease of creatine content of muscle. As the difference seemed to be one of slower action in my case, I made also the experiment, in which 0.5 cc. of 1% atropin sulphate was injected into right dorsal muscle, and after a little rubbing, allowed to stay in water and creatine content of muscle of left dorsal muscle was determined. The result is shown in Table VII.

Table VII.

The effect of atropin injected into the dorsal muscle on the creatine content of carps. 1/100 atropin 0.5 cc. 20°C water temp.

Time	Creatine content		Increase or decrease	
	Control	Experiment.	Absolute	Percentage
min.	*mg/dl*		*mg/dl*	
5	453	421	−32	−7.0
„	„	435	−17	−3.8
30	„	445	− 8	−1.8
„	„	„	− 8	−1.8
60	„	400	−53	−11.2

As can be seen from the table, atropin injected causes the decrease of creatine content of muscle, together with this the fish falls into the state of paralysis.

VI. The action of insulin applied through the gill.

Several authors (Noble and Macleod, 1923, Sordelli, Houssay and Mazzocco, 1923; Houssay and Rietti, 1924) stated that the injection of insulin causes neither a decrease or even a slight increase of blood sugar content in case of fish, differing entirely from the effect on homoiothermic animal. Root, Hall and Gray (1931) reported that 10 units of insulin, injected to *Stenotomus chrysops* (Scup) weighing 250 gm. caused hyperglycemia for about the first 60 minutes, to decrease slowly and to

fall into hypoglycemia. Interested to know if insulin is easily absorbed through the gill, and furthermore, considering that the application of insulin through the gill may give a result different from that by injection, the following experiments were performed.

As already stated above under the section of adrenalin, the blood sugar content of fish is quite variable. It is therefore indispensable that we must carry on the work under definite conditions. To attain the same state of nutrition, carp obtained from the fish-pond were fasted for a certain period. At the same time the change of sugar content of blood during the fasting period was followed. The amount of blood sugar was determined by the Hagedorn-Jensen method with 0.05 cc. of blood.

The result is shown in Table VIII and Fig. 6.

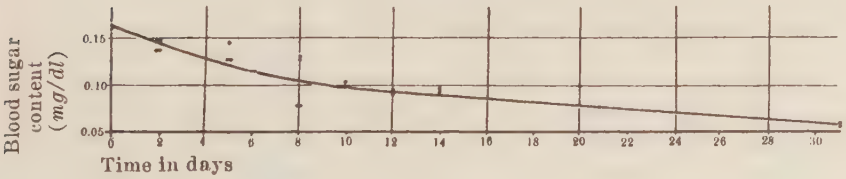
TABLE VIII.

The effect of starvation on the sugar content of blood of carps.

Days	1	2	5	6	7	8	10	12	14	31
Sugar Content *mg/dl*	0.163	0.146	0.146	0.111	0.111	0.079	0.097	0.084	0.092	0.054
	0.163	0.146	0.122	0.111	0.122	0.079	0.104	0.092	0.097	0.057
	—	0.138	0.122	—	—	0.131	—	—	—	—
	—	0.136	—	—	—	0.132	—	—	—	—
Average	0.163	0.141	0.130	0.111	0.116	0.105	0.100	0.088	0.094	0.055

Fig. 6.

The effect of starvation on the sugar content of blood of carps. (from April 5–May 14)



The table and figure indicate that the sugar content tends to decrease during starvation. It is therefore quite necessary that we determine the blood sugar of the control in the same nutritional condition with the experimental animal.

1. 5 units of insulin were added to 5 liters of water, in which a carp had been previously placed for 30 minutes, and after a certain definite time the content of blood sugar was determined. The carps remained very quite throughout all these experiments.

The result is shown in Table IX and Fig. 7.a.

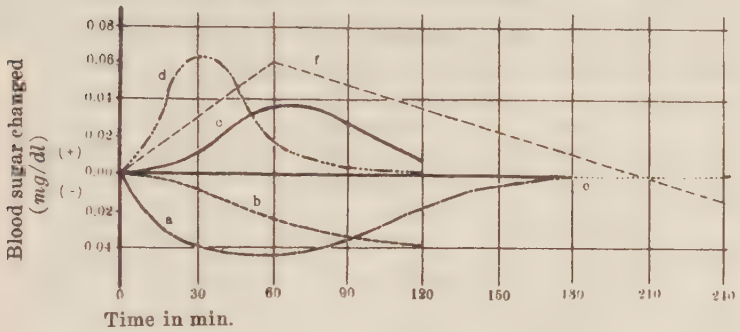
As can be seen from the table and figure insulin causes the decrease of blood sugar, the minimum of which lying at 60 minutes after the application of insulin, and the normal value returns about two hours later. This result is entirely against the result obtained by former authors as above mentioned, in which insulin always calls forth hyperglycemia at least in the beginning. As it seems

Table IX.

The effect of insulin in the water on the blood sugar of carps (5 units of insulin in 5*l* of water temp. 20°C.)

Time	Experiment	Control	Difference	Diff. ave.
	Blood sugar	Blood sugar		
min.	*mg/dl*	*mg/dl*		
5	0.083	0.104	−0.021	
„	0.079	„	−0.025	
„	0.081		−0.028	−0.023
30	0.110	0.141	−0.031	
„	0.070	0.111	−0.041	
„	0.081	„	−0.030	−0.034
60	0.079	0.132	−0.053	
„	0.101	0.131	−0.031	
„	0.088	0.122	−0.034	
„	0.077	„	−0.045	
„	„		−0.045	−0.041
120	0.083	0.104	−0.021	
„	0.079	„	−0.025	
„	„		„	
„	0.088		−0.016	−0.022
180	0.086	0.088	−0.002	
„	0.079	„	−0.009	−0.006

Fig. 7.
The effect of insulins which were treated by various conditions on the blood sugar of carps.



a —·—·—·— Insuline only.
b ———— Dialysate.
c ———— Partly dialysed insuline.
d ——·——·—— Thoroughly dialysed insulin.
e ———— Insulinized tank water after 3 hrs tank water after.
f ———— Root's experiment (injected).

to us that the difference between ours and those of other authors may be caused by the method of application, I have dialysed the insulin, with the view in mind to know whether easily dialysable part has a different action from that of a hard dialysable one.

2. 50 units of insulin contained in 5 cc. were dialysed for 2 days against 10 cc. of distilled water. Both 1 cc. of dialysate and 0.5 cc. of content remaining in collodion sack were tested separately. The result with dialysate was given in Table X and Fig. 7.b.

Table X.
The effect of dialysate of insulin on the blood sugar of carps.
20°C water temp.

Time	Experiment	Control		Difference
	Blood sugar	Blood sugar	Average	
min.	*mg/dl*	*mg/dl*		
30	0.124	0.129	—	−0.005
„	0.119			−0.010
60	0.081	0.101	—	−0.020
„	0.077			−0.024
„	0.127	0.154	0.150	−0.023
„	„	1.146		−0.023
120	0.047	0.084	0.088	−0.041
„	0.056	0.092		−0.032

The table and figure indicate that the dialysate shows the same hypoglycemic action as whole insulin preparation, though its action seems to be less intense. On the other hand the part remaining within the collodion sack shows a rather hyperglycemic action as Table XI and Fig. 7. c clearly indicate.

Table XI.
The effect of dialysed insulin in the water on the blood sugar of carps 20° water temp.

Time	Experiment	Control		Difference
	Blood sugar	Blood sugar	Average	
min.	*mg/dl*	*mg/dl*		
30	0.081	0.083	0.082	−0.001
„	0.088	0.081		+0.001
„	„			„
60	0.184	0.146	0.146	+0.038
„	„	„		„
120	0.106	0.097	0.095	+0.011
„	„	0.092		„

In this case the dialysable substance still remains in an amount of one third of the original and may act partly against hyperglycemic action. I have therefore tried thoroughly to get rid of the dialysable substance by the next experiment.

3. 50 units of insulin were placed within a collodion sack and were dialysed against running distilled water. The resulted non dialysable content of collodion sack shows more intense hyperglycemic action than that of Fig. 7. c curve, as can be seen from Table XII and Fig. 7.d indicate.

These three experiments tell us the fact that insulin can be divided into two fractions by dialysis, one of which lowers the blood sugar content, while the other elevates the same.

4. In all of the previous experiments the blood sugar content returns to the normal value after 3 hours. To decide whether this is caused by the disappearance of insulin from the solution or not, an experiment was made, in which a carp was placed in 5 liters

Table XII.

The effect of non-dialysable portion of insulin obtained by thorough dialysis on the blood sugar of carps.

Time	Experiment	Control		Difference	Diff. ave.
	Blood sugar	Blood sugar	Average		
min.	*mg/dl*	*mg/dl*		*mg/dl*	
20	0.093	0.054	0.055	+0.038	+0.038
30	0.117	0.057		+0.062	+0.060
„	0.113			+0.058	
60	0.072			+0.017	+0.016
„	0.070			+0.015	
120	0.056			+0.001	0.000
„	0.054			−0.001	

of water containing 5 units of insulin and after 3 hours a new carp replaced the old one. As Table XIII indicates, insulin almost disappears from the solution by the presence of carp during 3 hours Fig. 7.e.

5. The fractions obtained by dialysis stated above were then used to test their actions when they were injected. 0.5 cc. of each of these fractions was injected into the right dorsalateral muscle and the blood sugar was determined after 5, 15, 30, 60 and 90 minutes. The results are shown in Table XIV and Fig. 8.

As can be seen from the table and figure the injection of the dialysate causes the hypoglycemia throuthout these periods, while

Table XIII.

The effect of insulinized tank water after 3 hours stying of a carp on the blood sugar content of another carp.

Time	Sex	Experiment		Sex	Control			Difference
		Body length	Blood sugar		Body length	Blood sugar	Average	
min.		cm	*mg/dl*		cm	*mg/dl*		
60	♀	09.6	0.125	♀	18.4	0.122		+0.003
—	—	—	—	„	„	„	0.122	—
„	♂	20.6	0.115	„	19.2	0.111		+0.004
„	„	„	0.111	„	„	„	0.111	±0.000

Table XIV.

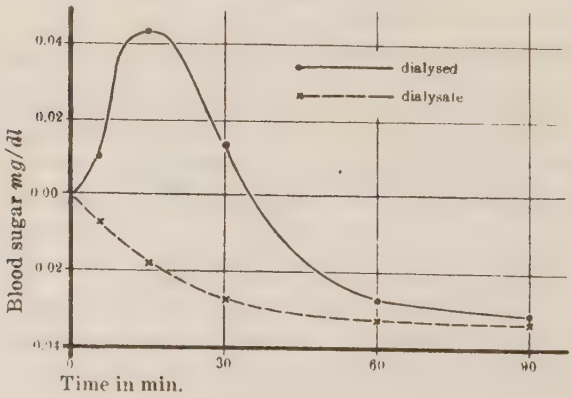
The effect of insulin (dialysed and dialysate) injected into the dorsal muscle on blood sugar of carps.

Experiment (dialysed)		Control	Difference	Average
Time of exp.	sugar *mg/dl*	sugar *mg/dl*		
min.				
5	0.139	0.129	+0.010	+0.010
„	„	0.130	+0.009	
15	0.173	0.129	+0.044	
„	„	0.128	„	+0.044
„	0.172		+0.043	
„	0.174		+0.045	
30	0.141	0.127	+0.014	+0.014
„	„	„	„	
60	0.100	0.127	−0.028	−0.029
„	0.099	0.128	−0.029	
90	0.096	„	−0.032	−0.032
„	„	„	„	

Experiment (dialysate)		Control	Difference	Average
min.				
5	0.117	0.125	−0.008	−0.008
15	0.108	„	−0.017	−0.018
„	0.107		−0.018	
30	0.098	0.127	−0.029	−0.029
„	„	0.125	„	
60	0.094		−0.033	−0.033
„	0.095		−0.032	
90	0.094		−0.033	−0.033

Fig. 8.

The effect of injection of insulin (dialysed and dialysate) into the dorsal muscle of carps.



that of dialysate insulin called forth the hyperglycemia within the first 30 minutes and the blood sugar shifts to the hypoglycemic state. This result coincides with those obtained in water tank experiment as are shown in Table IX, X, XI, XII and Fig. 7.a, 7.b, 7.c, and 7.d, and fortify the belief that insulin is composed of two components, the dialysable portion of which always causes the hypoglycemic action, while non dialysable fraction causes the hyperglycemic reaction in the beginning.

VII. The action of urease and its trypsic digest through the gills.

Sumner (1924, 1925) isolated a crystalline globulin with usease activity and found also the loss of its activity by the trypsic digestion. He insisted therefore on the proteic nature of urease. Waldschmidt-Leitz and Steigerwaldt (1931), however, were unable to found the disappearence of enzymic activity on trypsic digestion and considered the protein only as the material on which the urease is absorbed. To get a clearer idea on this point some experiments concerning the action of urease and its trypsic digest were made, the results of which are being given in the following.

1. The change of the ammonia content of blood of carp, when urease is added to the tank water.

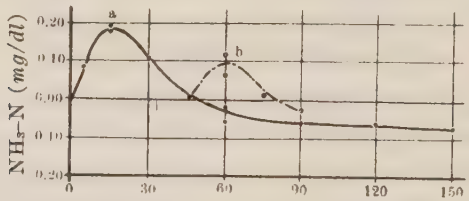
1. 1 gm. of urease (Merck) was added to 5 liters of water, in which a carp had been kept for 30 minutes, and after the lapse of several intervals the amount of ammonia in blood was determined by the Folin method (1932). As the ammonia content of blood may vary under several conditions, sufficient care was taken to attain always the same condition and the determination with normal control was always provided. The result is shown in Table XV and Fig. 9.a.

As can be seen from the table and figure the amount of ammonia increases directly after the addition of urease to the water tank, to attain its maximum at 15 minutes and to decrease slowly. When the content of ammonia returns to normal value, the second 1 gm. of urease was added again. The second rise in

Table XV.
The effect of urease on the blood NH_3-N of carps
(1 gm. in 5 *l* water 20°C water temp.)

Time	Experiment	Control	Difference
	NH_3-N	NH_3-N	
min.	*mg/dl*	*mg/dl*	*mg/dl*
5	0.48	0.39	+0.09
15	0.58	—	+0.19
„	0.59	—	+0.20
30	0.55	0.43	+0.11
45	0.40	0.39	+0.01
60	0.39	0.40	−0.01
„	0.33	0.41 } 0.39	−0.60
„	—	0.37 }	—
90	0.33	0.39	−0.06
120	0.33	„	−0.06
150	0.25	0.32	−0.07

Fig. 9.
The effect of urease on the amount of blood NH_3-N of carps
(1 gm. of urease added to 5 *l* of water. temp. 20°C).



ammonia content of blood was again observed. The degree of the rise was far inferior to that of the first rise, as Table XVI and Fig. 9.b. indicate.

This increase of blood ammonia does not mean, however, the direct evidence of absorption of urease through the gill, because the urea, which had been excreted by the carp during 30 minutes previous to the addition of urease, may be decomposed to ammonia by urease outside of fish and absorbed as ammonia and causes the

Table XVI.

The amount of blood ammonia at several intervals after the renewal addition of 1 gm. of urease to the carp water tank of 5 liter capacity, in which 1 gm. of urease was put 45 minutes previous to the experiment.

Experiment		Control	
Time	NH_3-N	NH_3-N	Difference
min.	*mg/dl*	*mg/dl*	*mg/dl*
15	1.41	1.29	0.12
„	1.38	1.32	0.06
35	1.33	1.32	0.01
40	1.29	„	−0.03

increase of blood ammonia.

To decide how far urea is excreted into the tank water by a carp for 30 minutes, and how much blood ammonia may be increased by the absorption of ammonia produced from this excreted urea by the urease added, the following experiment was undertaken.

2. A carp was kept in 5 litrers of water, well aerated, for 7–24 hours. 200 cc. of this tank water were used for the estimation of urea. As Table XVII indicate a carp excretes 0.802 mg. Urea-NH_4-Nitrogen per hour.

Table XVII.

Ammonia nitrogen excreted by carps.
(20°C water temp. in 5 *l* of water.)

No.	Time	Body length	Sex	NH_3-N excreted	NH_3-N 1 hr
	h	cm		*mg/dl*	*mg/dl*
I	7 30′	23.9	♂	4.5	0.60
II	7	23.5	♀	10.1	1.44
III	24	25.2	„	14.3	0.595
IV	„	26.0	♂	15.7	0.654

Average.....0.802

Assuming that this amount of urea-NH_3-nitrogen is converted into ammonia by the urease added to tank-water and absorbed by

the fish, the amount of possible increase of blood ammonia content was considered. As namely 0.401 mg. Urea-NH_3-nitrogen, excreted for 30 minutes, corresponds to 1.2 mg. NH_4Cl, 0.6 mg. NH_4Cl was added to tank-water, in which a carp had been kept, and the amount of blood ammonia was determined after 5 minutes. As can be seen from Table XVIII and XIX the addition of 0.6 mg. NH_4Cl, half an amount of possible total conversion of excreted urea to ammonia, calls forth the increase of blood-ammonia by 0.174 *mg/dl* at 5 minutes after the addition of the salt. This result indicates to us that the increase of blood ammonia occurring after the addition of urease to the tank water may be duely caused by the absorption of converted ammonia outside of fish from excreted

Table XVIII.
Normal content of ammonia nitrogen in blood of carps.

Sex	Body weight	Body length	Blood NH_3-N
	gm.	cm.	mg/dl
♂	390	23.4	0.366
♀	410	24.5	0.372
♂	295	21.5	0.345
♂	350	22.8	0.425

Average.....0.377

Table XIX.
The amount of ammonia nitrogen in blood of carp at 5 minutes after the addition of 0.6 mg NH_4Cl to the tank-water.

Sex	Body weight	Body length	Blood NH_3-N
	gm.	cm.	*mg/dl*
♂	395	22.7	0.453
♀	415	22.3	0.605
♂	370	22.7	0.590

Average.....0.551

urea, and not by the absorption of urease through the gill into the blood.

3. The conclusion obtained in the preceding section, that

urease may not be absorbed through the gills, can be fortified by the following experiment. 250 cc. of 1% urease solution were dialysed through an animal membrane against 250 cc. of distilled water for 12 days. The dialysate showed, however, no trace of urease activity. Then the ultrafiltration through collodium membran and Zsigmondy-Bachmann's membrane filter No. 60 was tried. Both filtrate were water clear 3 cc. of the ultrafiltrate obtained from 2 per cent urease solution were added to 3 cc. of 1 per cent urea solution, and the activity was determined by the Van Slyke Cullen method. The results are shown in Table XX.

TABLE XX.
The effect of ultra-filtration on urease content in the filtrate.

No. of exp.	Nature of filtrates	Titration value
		cc.
1	Colloid membrane filtration complete filtrate clear.	25.0
2		"
3		"
4	Zsigmondy-membrane No. 60 filtration complete. filtrate clear.	24.1
5		"

As can be seen from the table the collodion membrane filtrate contains no trace of urease, while Zsigmondy membrane filtrate was slightly active. Collodion membrane and Zsigmondy membrane were both permeable to methylen blue, congored and ponceau, with the difference that the latter membrane is more easily permeable. From the results of these experiments we can assume that the urease (Merck) used can not be absorbed through the gills.

2. *The effect of trypsin digest of urease on the blood ammonia content of fish.*

50 cc. of the trypsin digest of urease, obtained by digesting 100 cc. of 2 per cent urease solution with 20 cc. of 1 per cent trypsin (Merck) at pH 8.0, were taken out at several intervals, and added to the tank water, in which a carp had been kept for 30 minutes,

and after 15 minutes the content of ammonia of blood was determined. The result is shown in Table XXI and Fig. 10.

The table and figure indicate that partly digested urease still retain its activity, while a long digested one loses its activity entirely.

This disappearence of activity of trypsin digest of urease on increasing blood ammonia of carp kept in water containing trypsin

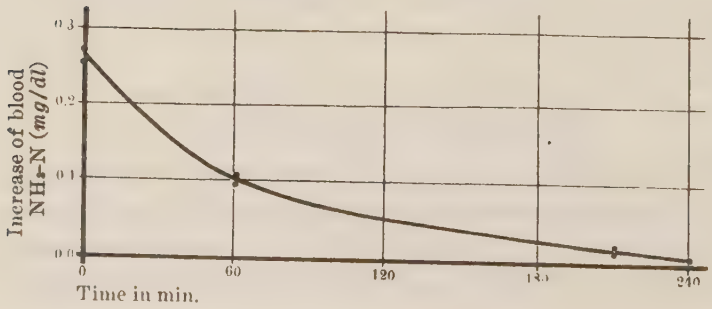
Table XXI.

The effect of urease treated by trypsin on the ammonia nitrogen of blood (50 cc. of 2% urease were treated by 10 cc. of 1% trypsin and ammonia determined after 15 min.)

Time	Increase of Blood NH_3-N	Average
min.	mg.	
at once	0.275	0.265
„	0.255	
60	0.097	0.985
„	0.100	
210	0.010	0.008
„	0.006	
„	0.011	
„	0.005	
240	0.005	0.005

Fig. 10.

The effect of trypsin digest of urease on the blood ammonia content of fish.



digest, goes parallel with the activity of the digest on the urea *in vitro* as Table XXII a. b. c. and Fig. 11 indicate. Here 3 cc. of the digest were taken out at different intervals, then added to 3 cc. of 1 per cent urea, and their activity was determined by the Ván Slyke and Cullen method. Fig. 11.a. The control experiment of testing the activity of urease used, and that of possibility of ammonium production from urea (Fig. 11.c) and urease and trypsin, except urea was also conducted. (Fig. 11.b).

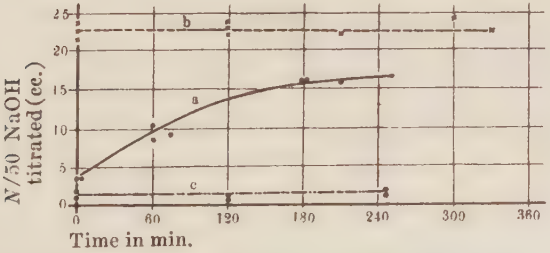
TABLE XXII.

The effect of trypsin digestion on the activity of urease.

	Experiment	Time in minutes and N/50 NaOH titrated								
		0	60	75	120	180	210	250	300	330
a	(Urease +trypsin)3cc. +urea 3cc.	3.85		8.47			16.10			
		3.81	10.61			16.20				
			8.31			16.20		16.55		
b	(Urease +trypsin)3cc.	23.04			23.15		22.45			
		22.95			22.70					22.68
		24.60			24.40				24.82	
c	Urease 3cc. +urea 3cc.	2.28			1.00			1.56		
		1.22			0.45			1.95		

Fig. 11.

The effect of trypsin on the activity of urease and control experiments.



3. The effect of lightly trypsin digested urease on the blood ammonia content of fish.

In this experiment urease was treated with trypsin at pH 7.0

for 1.5 hour, quantities of urease and trypsin being the same as the foregoing experiment. This urease solution remained still partly active. The effect of this partly digested urease on the blood ammonia content of fish followed as the foregoing experiment. The result is shown in Table XXIII and Fig. 12.

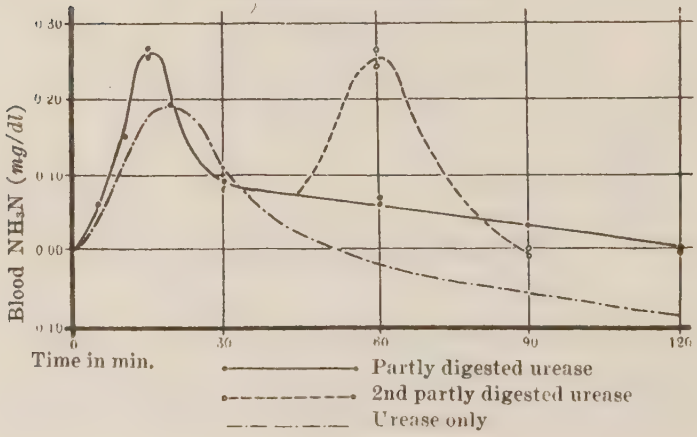
Table XXIII.

The effect of urease lightly digested by trypsin on the ammonia nitrogen of blood.

Time	Increase or decrease of blood NH_3-N
min.	mg./dl
5	0.06
10	0.15
15	0.27
„	0.26
20	0.19
30	0.08
„	0.09
60	0.09
„	0.08
90	0.03
120	−0.02
„	0.00

Fig. 12.

The effect of lightly trypsin digested urease on the blood ammonia content of carps.



We can see from the table and figure that partly digested urease is more active than the untreated urease in increasing blood ammonia content.

That this increased activity of partly trypsin digested urease in increasing blood ammonia is not due to the increased urease activity was verified by the *in vitro* experiment with digested urease and urea. It is therefore very likely that it was caused by increased permeability of enzym. To clear up this point the dialysate and ultrafiltrat of the partly trypsin digested urease were obtained and their *in vitro* urease activity was determined. As Table XXIV indicates partly digetsed urease is found to easily permeate membrane.

One more experiment was performed in which urease solution was filtered through Zsigmondy-Bachmann's membrane filter. Non filtrable portion was washed well with water, and digested partially with trypsin, the latter mixture being again ultrafiltered. When the first urease ultrafiltrate, washed urease residue, and ultrafiltrate obtained from the latter after treatment with trypsin were examined for their urease activity, we find the fact, as can be seen from Table XXV, that non ultrafiltrable urease portion becomes ultrafiltrable upon the partial digestion with trypsin.

Table XXIV.

In vitro activity of dialysate and ultra-filtrate of slightly digested urease (Urease solution pH 7.0)

No.	Amount of $N/50$ H_2SO_4 neutralized by NH_3		
	(Urease + trypsin) + Urea cc.	Ultra-filtrate cc.	Dialyzed urease* cc.
I	23.3	2.0	20.6
II	23.4	14.8	20.9
III	26.3	12.9	22.0

1) Collodium membrane, II. III. Zsigmondy Bachmann filter.

*) Animal membrane was used.

Table XXV.
Behavior of urease activity upon the action of trypsin
(Urease solution pH 7.0)

No.	Amount of $N/50$ H_2SO_4 neutralized by NH_3		
	Urease filtrate	Urease washing	(Urease + trypsin) filt.
	cc.	cc.	cc.
I	6.0	0.0	2.1
II	6.6	0.0	1.1
III	4.6	0.0	1.6

Zsigmondy-Bachmann's membrane filter was used.

4. The effect of injection with different fraction of urease on the ammonia content of carp.

All of the foregoing experiments indicate to us that the urease itself can hardly enter within the body of fish, although it decomposes the urea excreted by the fish and ammonia thus produced causes the increase of blood ammonia after being absorbed, and that partially trypsin-digested urease, through its gain in membrane permeability, more strongly increases the ammonia content of blood. What, then, will be the effect of intravenous injection of the urease, its trypsin digest, and the ultrafiltrate of the latter?

We took off a few scales at the left side near the caudal fin, exposed the caudal vein, injected the solution to be tested, covered the cut surface with collodion, put the fish into the tank, and determined the ammonia after a definite time.

The untreated urease was injected in an amount of 1 cc. of its 1 per cent solution per 200 gm. of body weight of the fish. The partially trypsin digest, composed of 25 cc. of 1 per cent urease solution and 5 cc. of 1 per cent trypsin and kept for 1.5 hour at 45° and pH 7.0, was used also in an amount of 1 cc. per 200 gm. The Zsigmondy-Bachmann membrane No. 60 filtrate of the latter was used in an amount of 1 cc. for 300 gm. body weight.

The results, shown in Table XXVI and Fig. 13, indicate that the

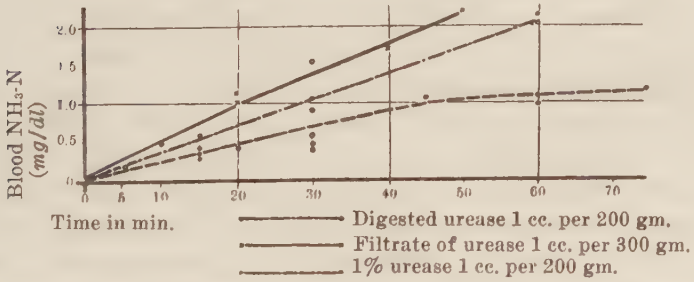
TABLE XXVI

Comparison of the increase of the blood ammonia content by the injection of differently treated urease fraction. The value is the mean of several experiments.

Time	Untreated urease	Partially digested with trypsin	Ultrafiltrate of trypsin-digest
min.	*mg/dl*	*mg/dl*	*mg/dl*
5	—	0.16	—
10	—	0.48	—
15	0.35	0.51	—
20	0.37	—	1.005
30	0.58	0.94	—
40	—	—	1.70
45	1.08	—	—
50	—	—	2.20
60	1.02	2.9	—
75	1.23	—	—

Fig. 13.

The effect of urease injected into the venous blood vessel of carps on the amount of blood ammonia.



partially trypsin-digested urease is more active than the untreated one, while the ultra-filtrate of trypsin-digest is far more active.

5. *On the stability of urease in the water tank.*

On the addition of urease or its partial trypsin digest to the tank water the increase of ammonia of carp blood attains its maximum at about 15 minutes of their action and tends to decrease. In considering its mechanism it is necessary to see whether the urease is inactivated by the secrete or excrete of the fish. This

looks quite probable because in the presence of fish, urease begins to agglucinate with the lapse of time, and to precipitate finally. To see how far this condition interferes with the activity of urease the following experiments were tried.

Each of 1 gm. of urease was added to 5 liters of tank-water,

i) only containing water alone,

ii) in which a carp had been kept for 30 minutes, and then was taken out,

iii) in which a carp had been kept for 30 minutes, and kept on in still longer after the addition of urease,

and the activity of urease was determined by Van Slyke-Cullen's method at various intervals. The results are shown in Table XXVII and Fig. 14.

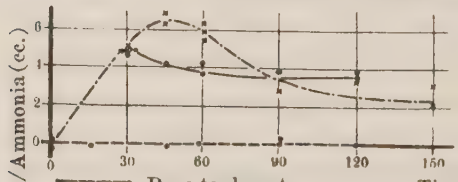
TABLE XXVII.

The activity of urease with an and without the excrete of the fish.

Condition of experiments	Amount of $N/50$ NaOH titrated (cc.)							
Time in min.	0	15	30	45	60	90	120	150
Urease only	14.2	14.1	—	—	—	13.7	—	—
	13.4	—	—	14.1	14.0	13.8	13.9	—
Excrete of carp accumulated for 30 min+urease			9.5	9.9	9.9	—	—	—
			9.2	—	10.6	—	10.6	—
			9.2	—	—	10.1	10.5	—
Carp+Urease			9.0	7.1	8.6	11.3	—	11.2
			9.1	7.5	7.8	—	—	12.3
			9.1	—	8.0	—	10.9	—

Fig. 14.

Amount of ammonia produced from tank-water by the action of urease.



Pure tank-water.

Tank-water previously retained a carp for 30 min.

Tank-water containing a carp throughout the experimental period.

We can see from the table and figure that the urease does not lose its activity much in tank water, when it is present alone, while the secrete and excrete of carp accumulated for 30 minutes considerably lessens, but does not totally lose the activity.

VIII. Summary.

1. Fishes are quite suitable test subjects for the biological action of many soluble and diffusible substances.

2. Easily permeable substance like saponin, adrenalin and atropin can be promptly absorbed, especially through the gills and effect their specific action.

3. Insulin can act through the gill. Perhaps it consists of easily dialysable and non dialysable fraction.

4. Urease seems to be impermeable to the gills. The partial trypsin digestion makes urease more permeable and accordingly becomes active when applied through the gills. On the prolonged trypsic digestion the urease action disappears entirely. The activity of urease is likely concerned with some chemical construction of polypeptid nature, destructable by trypsin, but not with colloidal structure as protein itself.

The work has been carried out under the direction of Prof. Dr. Samuro Kakiuchi, Director of Biochemical Institute of the Tokyo Imperial University, to whom the writer wishes to express his hearty thanks for the continuous guidance, precious advices and valuable criticisms given during the course of the study and also wishes to thank sincerely Prof. Dr. Shinkishi Hatai, Director of Biological Institute of Tôhoku Imperial University, who has encouraged the writer through out the study.

In this place the writer wishes to express thanks for financial aid given to *"the Foundation for the Promotion of Scientific and Industrial Research in Japan."*

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The Journal of Biochemistry, Vol. 23, No. 2.

ON THE EXISTENCE OF A SUBSTANCE WHICH CONTROLS THE SPLITTING AND SYNTHETIC ACTION OF LIPASE.

I. Report.

By

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(Received for publication, January 10, 1936).

It is usually assumed that enzyme can catalyse anabolic as well as catabolic processes in living cell. But little is known what factor does determine this dualistic action of an enzyme. Moreover, the strict reversibility of this action has not yet been proved on any enzyme. So far as we know, the plastein formation by pepsin is the typical example where the synthetic action of the enzyme has been substantially demonstrated. In this case pepsin can catalyse the reaction under the law of mass action. Namely when the concentration of the split-products is raised, it acts as a synthetic agent. But even in this case the reversibility in the strict sense can not be held. Thus it is justified to say that our present state of knowledge on the anabolic processes in the living organismus is extremely poor.

In this regard, the substance isolated from castor bean by the author offers a very interesting and important example in the enzyme chemistry. The substance is a white powder and readily oxidised in the air. In the oxidised state it accelerates the synthetic action of castor bean lipase, while in the reduced state the splitting action of the same.

Also it can exist as an intermediary form, which neither accelerates nor retards both reactions. For the present we call this substance "lipase-activator". These forms may be transformed by each other according to the conditions prevailing in the

cell such as hydrogen ion concentration or redox potential. Therefore, by the state and the concentration of this substance which exists in an intimate connection with the enzyme in the seed the direction and the extent of the metabolism of fatty matter catalysed by the lipase is determined. No such a substance has yet been reported on any enzyme. If we are allowed to assume the existence of an analogical substance for other enzymes the explanation of the bewildering complexities of cell household may be fairly simplified.

Experimentals.

1. Preparation of Ricinus-lipase.

The enzyme preparation applied for this study was prepared from castor bean-seeds according to the method described by E. Takamiya (1935). 100 g of castor bean-seeds were crushed, and after the addition of 14 cc. *N*-sulphuric acid solution kneaded in a mortar thoroughly. By this acid treatment the lipase was freed from the protein. The whole material was put into a stoppered flask to which was gradually added 500 cc. of re-distilled ether under constant shaking and the passage of purified CO_2 gas allowed. As the ricinus-lipase is easily inactivated by oxygen, the whole procedure should be carefully carried out avoiding the contact with air. The etherial solution was submitted to centrifugalisation, upon which a milky ether extract was obtained. This was mixed with the same volume of re-distilled petroleum ether, and the whole was stored in a stoppered bottle overnight in an ice box. The supernatant portion was sucked off. Then 700 cc. of ether was added to the residual solution containing precipitates, shaken thoroughly and left overnight at room temperature. An upper clear phase was sucked off. This treatment with ether was repeated once more. The residual solution was poured into a centirfuging tube, sealed with stopper and centrifuged. The precipitate was washed twice with petroleum ether and dissolved in 30 cc. of olive oil. The somewhat turbid olive oil fraction was separated by centrifuging, and washed with ether and petroleum ether two times

respectively.

Since this lipase preparation is very unstable and easily inactivated by air, the test for enzymic activity and other investigations were carried out after being dissolved in olive oil under the protection from inactivation by air.

The enzyme preparation obtained was very active showing lipase value (pH L. W.) 47, which was determined according to the method of Willstätter and Waldschmidt-Leitz (1924).

2. *Preparation of "lipase-activator".*

The procedures up to the first addition of petroleum ether were the same as described in the above section. The ether-petroleum ether mixture was transfered into a stoppered bottle and placed in a refrigerator overnight. The resultant precipitate was collected by centrifuge, and extracted three times with ether, each time using 30 cc. To the combined ether extract the same volume of petroleum ether was added and the solution was placed in a refrigerator for at least two days, the upper clear portion was decanted off and the rest of solvent was expelled by passing through purified CO_2 gas at room temperature.

Thus the white powder with rhombic crystals was obtained, which is the reduced form of "lipase-activator". It can be transformed readily into the oxidised form by exposing to air or by treating with diluted hydrogen peroxide.

The intermediary form, which seems to be partially oxidised was also obtained by drying the ether-petroleum ether solution which contains the reduced form in vacuum desiccator over H_2SO_4.

The substance was dissolved in olive oil. If there exists undissolved portions it was centrifuged off and upon the clear solution the effect was investigated.

3. *Experimental Results.*

(a) Hydrolytic Reaction.

A definite amount of the enzyme-olive oil solution prepared as above mentioned, was pipetted into a glass vessel of the size

3.5 cm × 14.0 cm, and mixed with lipase activator-olive oil solution. To this 2 cc. of $N/2$ acetic acid-ammonium acetate mixture (pH 4,7) and a certain amount of olive oil as shown in the table were added. The whole was mixed thoroughly and placed in the thermostat kept at 30°C for 30 minutes. At the end of this time the contents were transfered into a conic flask by means of 30 cc. ethylalcohol and 15 cc. ether and the liberated free acid was titrated with $N/10$ KOH-alcoholic solution using phenolphthalein as an indicator.

Table I.

Experiment on the reduced form of "lipase-activator".

Lipase preparation in cc.	Olive oil in cc.	Substance added in cc.	Acetate buffer solution of pH 4.7 in cc.	Degree of hydrolysis in %.
2	1	0	2	19.2
2	0	1	2	25.6
2	0.5	0.5	2	22.3
2	0.8	0.2	2	21.2
0	2	1	2	0

Table II.

Experiment on the oxidised form of "lipase-activator".

Lipase preparation in cc.	Olive oil in cc.	Substance added in cc.	Acetate buffer solution of pH 4.7 in cc.	Degree of hydrolysis in %.
2	1	0	2	19.2
2	0	1	2	6.2
2	0.5	0.5	2	9.5
2	0.8	0.2	2	12.8
0	2	1	2	0

Table III.

Experiment on the intermediary form of "lipase-activator".

Lipase preparation in cc.	Olive oil in cc.	Substance added in cc.	Acetate buffer solution of pH 4.7 in cc.	Degree of hydrolysis in %.
2	1	0	2	17.8
2	0	1	2	17.6
2	0.5	0.5	2	17.8
2	0.8	0.2	2	17.7
0	0	1	2	0

(b) Synthetic Reaction.

The synthetic reaction was studied in the system containing oleic acid, glycerol, acetate buffer-solution, ricinus-lipase and "lipase-activator". They were mixed well at the beginning of the experiment. The vessel used here was the size of 3.5 cm × 14.0 cm, the reaction temperature was 25°C and the reaction time was 7 hours.

At the end of this time the oleic acids which remained unsynthesized were titrated as described in the foregoing experiment.

TABLE IV.
Experiment on the reduced form of "lipase-activator".

Lipase preparation in cc.	Oleic acid in cc.	Glycerol in cc.	Acetate buffer solution of pH 4.7 in cc.	Substance added in cc.	Degree of synthesis in %.
2	3	4	2	0	12,4
2	3	4	2	1	3.1
2	3	4	2	0.8	3.9
2	3	4	2	0.5	5.2
0	3	4	2	1	0

TABLE V.
Experiment on the oxidised form of "lipase-activator".

Lipase preparation in cc.	Oleic acid in cc.	Glycerol in cc.	Acetate buffer solution of pH 4.7 in cc.	Substance added in cc.	Degree of synthesis in %.
2	3	4	2	0	12.4
2	3	4	2	1	19.7
2	3	4	2	0.8	19.3
2	3	4	2	0.5	17.3
0	3	4	2	1	0

TABLE VI.
Experiment on the intermediary form of "lipase-activator".

Lipase preparation in cc.	Oleic acid in cc.	Glycerol in cc.	Acetate buffer solution of pH 4.7 in cc.	Substance added in cc.	Degree of synthesis in %.
2	3	4	2	0	12.4
2	3	4	2	1	12.3
0	3	4	2	1	0

From these experimental results it is evident that the reduced form of this substance acts as an activator on the hydrolytic reaction of ricinus-lipase and as an inhibitor on the synthetic reaction. The oxidised form acts as an activator for the synthesis and as an inhibitor for the hydrolysis. The intermediary form is indifferent for both hydrolytic and synthetic reaction.

Summary.

1) A substance has been isolated from castor-seeds, which controls the reversible action of lipase.

It has three forms, the reduced, the intermediary and the oxidised.

2) The reduced form of this substance, which seems to be the predominating form in castor bean-seeds, acts as an activator on the hydrolytic reaction of the ricinus-lipase and as an inhibitor on the synthetic reaction.

3) The oxidised form, which is easily obtainable from the reduced form by the auto-oxidation in air acts as an activator for the synthesis and as an inhibitor for the hydrolysis.

4) The intermediary form is indifferent for both synthetic and hydrolytic reaction.

Investigation on this substance will be continued.

The author is greatly indebted to Prof. Dr. K. Kodama for his kind criticisms and suggestions throughout this research.

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The Journal of Biochemistry, Vol. 23, No. 2.

ON β-GLUCURONOSIDASE.

III. Report.

By

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(Received for publication, January 14, 1936)

The purification of the enzyme.

In the previous communication of this series the author reported that the β-glucuronosidase of Masamune, which can split only β-glucuronide, is most abundant in grandular organs such as spleen, ovary, thymus, suprarenals and thyreoidea.

In this communication the method of extraction of the enzyme and its purification is reported.

Experimentals.

I. Extraction of the enzyme.

Bullspleen was used exclusively.

The fresh organ was freed from blood by pressing out and macerated into paste by chop machine.

For the extraction of the enzyme autolysis method or chloroform method were tried and the efficacy was compared.

The procedure of autolysis method is the same as reported by Masamune (1934), namely, one portion of the tissue pulp was shaken with double weight of physiological salt solution containing toluene in the proportion of 0.3 cc. to 10 cc. of the solution.

The whole mixture was kept in stoppered bottle and subjected to autolysis by incubating at 37°–38°C for different lengths of time.

At the laps of the time the solution was centrifugalised and the supernatant fluid (I. Extract) was taken for further purifications.

The second method is the use of chloroform for extraction, namely the tissue pulp was ground with geasand mixed with 3 times weight of chloroform water, placed in ice-chest for 24 hours and then the extract was separated by centrifugalisation.

Both extracts were treated as follows.

To the above crude extract the calculated amount of diatomaceus earth (in the ratio of 0.13 g to 10 cc. of the extract) was added, well shaken and then centrifugalised off. To this supernatant fluid (II. Extract) three times volume of 95% alkohol was added, the precipitate resulted was collected on a filter paper, the adherent alcohol was pressed off, suspended again in water of one third volume of the initial extract and kept in a refrigerator for 12–48 hours.

Then the suspension was acidified with 1*N* sulfuric acid in the ratio of 0.1–0.2 cc. to 10 cc. and again submitted to centrifugalisation. The enzyme is precipitated by retreatment with alcohol, removed from alcohol by rapid drying in vacuo over phosphorus pentaoxide and dissolved finally with half volume of water of the preceding extract. The solution thus obtained being faintly coloured brownish yellow.

On the solution the enzyme activity was determined as follows:—

Into the test tube of 40 cc. capacity with a ground stopper 1.0 cc. of *M*/15 Na-mentholglucuronate solution, 2.0 cc. of the enzyme solution and 2.0 cc. of *M*/5 acetate buffer (pH 5.2) were pipetted and one drop of toluene was added to the mixture.

After the test tube was placed in the thermostat of 38°C for certain intervals of time as indicated in the table the content was neutralised by 1.0 cc. *N*/5 NaOH, deproteinised by the addition of 4 cc. of 5.3% phosphotungustic acid and then free glucuronic acid was determined by Masamune's method (1933). The results are summarised in table I.

As indicated in table I the autolysis method gives the highest yield at 72 hours incubation and seems more effective than the chloroform water method, so that in the succeeding experiments the former method was used exclusively.

Table I.

enzyme sol	2.0 cc.
M/5 acetate buffer (pH 5.2)	2.0 cc.
M/15 Na-mentholglucuronate	1.0 cc.
toluene	1 drop

Autolysis method	Time of incubation (h)	24	48	72	96	120
	Glucuronic acid set free. (mgm)	4.94	6.37	8.35	8.32	8.27
Chloroform water method	Time of keeping in ice-chest (h)	24				
	Glucuronic acid set free (mgm)	5.98				

Incubation 38°C for 8 h.

The tissue pulp employed was from one and the same source in each method.

II. Further purification of the enzyme.

For the further purification of the enzyme various adsorption methods of Willstätter were tried.

A) The adsorption of enzyme by kaolin at various pH.

Calciumphosphate and Alumina B were found to adsorb the enzyme from the acidified extract almost completely but the elution in alkaline medium was less satisfactory.

On the other hand kaolin was found to adsorb the enzyme in acidic solution and set free in alkaline.

One of the typical experiments is given here.

Into a series of the centrifuge blasstubes of 60 cc. capacity, 10 cc. of the enzyme solution were pipetted, added to with 2.0 cc. acetate buffer (*M*/5) of various pH, and finally with 2.0 g of purified kaolin.

After being well shaken for 15 minutes kaolin was separated by centrifugalization and from the clear supernatant solution 10 cc. was pipetted out, adjusted to pH 5.2 by adding acetic acid or sodium acetate and made up to 11 cc. with water. On this solution, the enzyme remaining unadsorbed was determined.

The results are indicated in table II.

Table II.

supernatant or control enzyme sol.	3.0 cc.
M/15 Na-mentholglucuronate	1.0 cc.
M/5 acetate buffer (pH 5.2)	2.0 cc.
toluene	1 drop

Control enzyme sol. (original enzyme sol. diluted with dist. water 12/10×11/10 times)

pH	Gl. acid set free. x (mgm)	Activity of adsorbed enzyme. $a-x$ (mgm)	Adsorbed enzyme. $\frac{a-x}{a}\times 100$ (%)	Total N in supernatant 100 cc. (mgm)	Adsorbed total N in enzyme sol. 100 cc. by kaolin (mgm)	Adsorbed N (%)
2.6	0.00	5.99	100	1.45	18.35	93
3.6	0.00	5.99	100	1.62	18.18	92
4.4	0.15	5.84	98	2.27	17.53	89
5.0	0.92	5.17	85	2.53	17.27	87
5.6	4.17	1.82	31	5.70	14.10	75
6.0	4.75	1.24	21	7.21	12.59	64
6.6	5.21	0.78	13	9.60	10.20	52
7.2	5.46	0.53	9	9.82	9.98	51
control	$a=5.99$			19.80		

Incubation at 38°C for 5 h.

As is apparent from the table the adsorption of the enzyme is complete at pH 2.6–4.4 and becomes less and less towards more alkaline side.

The author also determined the amount of *N* adsorbed and calculated the percentage adsorption as given in rows VI and VII.

Of course the adsorption of enzyme run parallel to that of *N*.

B) In the next experiment the minimal amount of kaolin to adsorb the enzyme completely was measured.

The procedures are the same as in the foregoing experiment except that the acidity was fixed to the optimal pH 3.6 and that the amounts of kaolin varied from 0.2 g to 1.8 g.

The results are given in table III.

It was found that under the conditions of the above experiment the minimum amount of kaolin for complete adsorption is 1.4 g.

Table III.

supernatant or control enzyme sol.	3.0 cc.
$M/15$ Na-mentholglucuronate	1.0 cc.
$M/5$ acetate buffer (pH 5.2)	2.0 cc.
toluene	1 drop

Control enzyme sol. (original enzyme sol. diluted with dist. water $12/10 \times 11/10$ times)

Kaolin (gm.)	Gl. acid set free. x (mgm)	Activity of adsorbed enzyme. $a-x$ (mgm)	Adsorbed enzyme. $\frac{a-x}{a} \times 100$ (%)	Total N in supernatant 100 cc. (mgm)	Adsorbed total N in enzyme sol. 100 cc. by kaolin (mgm)	Adsorbed N. (%)
0.2	7.63	0.10	1	15.68	5.72	27
0.4	7.35	0.38	5	12.25	9.15	43
0.6	6.81	0.92	12	10.25	11.15	52
0.8	5.51	2.22	29	8.81	12.59	58
1.0	3.73	4.01	52	8.06	13.34	62
1.2	0.72	7.01	91	6.47	14.93	70
1.4	0.09	7.64	100	5.71	15.69	73
1.6	0.00	7.73	100	4.56	16.84	79
1.8	0.00	7.73	100	4.20	17.20	80
control	$a=7.73$			21.40		

Incubation at 38°C for 5 h.

Here 73% of N was also adsorbed.

C) The elution by sodium phosphate.

Each 15 cc. of the enzyme solution were treated with 2.4 g kaolin at pH 3.6 in centrifuge tube as in the above experiment. The unabsorbed upper layer was decanted off. Then various amounts of $m/15$ Na_2HPO_4 solution were added to it, and finally water up to the amount of 18 cc.

After being shaken for 30 minutes at a room temperature the eluate was separated by centrifugalisation.

On this solution the enzyme activity and its N-contents were determined. The results are given in table IV.

As will be seen from the table the highest recovery of the enzyme by sodium phosphate is nearly 60%. From the point of

Table IV.

eluate or control enzyme sol.	3.0 cc.
M/15 Na-mentholglucuronate	1.0 cc.
M/5 acetate buffer (pH 5.2)	2.0 cc.
toluene	1 drop

Control enzyme sol. (original enzyme sol. diluted with dist. water. 12/10 × 11/10 times)

M/15 Na_2HPO_4 sol. (cc.)	Gl. acid set free. (mgm)	Recovered enzyme. (%)	Total N in eluate 100 cc.		pH
			(mgm)	(%)	
1.0	0.29	4	2.01	8	4.8
1.5	0.68	10	3.81	16	5.86
2.0	2.91	43	4.90	20	6.56
3.0	3.66	54	6.13	25	6.94
4.0	3.99	59	7.22	30	7.16
5.0	4.12	61	7.67	32	7.23
6.0	4.14	61	6.90	30	7.32
7.0	4.32	63	8.16	34	7.40
Control	6.79		24.24		

Incubation at 38°C for 5 h.

view of *N*-contents, this being reduced to 30% the enzyme is concentrated two times.

D) Dialysis.

Finally the eluate was submitted to dialysis against destilled water, using collodium sack and a clear slightly yellowish solution was obtained. This was used in the succeeding experiments as purified preparation, which could be kept unimpaired for several weeks when kept in the ice-chest.

E) Some chemical properties of the solution are as follows.

Biuret Reaction	+
Millon's Reaction	−
Hopkins Cole Reaction	+
Nitroprussid Reaction (for SH radical)	−
PbS Reaction	+
Orcin Reaction (for pentose)	±
Phosphorus	+

It seems true that the enzyme is of protein nature.

Summary.

The method of extractions of β-glucuronosidase (Masamune) from bull's spleen and its purification by kaolin adsorption is given.

IV. Report.

Kinetics of β-glucuronosidase and the effects of various factors upon its activity.

Using the purified enzyme prepared in the manner as mentioned in the foregoing report the author investigated various factors which control the activity of the enzyme.

I. The effect of H-ion concentration.

In this respect Masamune (1933) carried out the experiment using citrate buffer and came to the conclusion that the optimal pH exists in the range between 5.2–5.6.

In the case of my experiment where acetate buffer was used it was found to be 5.0–5.2 as shown in table V.

Table V.

M/15 Na-mentholglucuronate .	1.0 cc.
M/5 acetate buffer (pH 4.6–6.6)	2.0 cc.
Enzyme sol.	3.0 cc.
toluene	1 drop

pH	4.6	5.0	5.2	5.6	6.0	6.6
Glucuronic acid set free. (mgm)	1.81	2.07	2.09	1.77	1.34	0.78

Incubation at 38°C for 8 h.

The discrepancy from Masamune's data might be sought to the effect of protein, which was reduced to one third in the purified enzyme.

II. The kinetics of the enzyme action.

In the next experiment the velocity of the decomposition of menthol-glucuronic acid by the enzyme was determined and the velocity constant was calculated according to monomolecular, bimolecular and Schütz's formula.

Into the conic flask with a stopper of 200 cc. capacity 36 cc. of purified enzyme solution, 24 cc. of *M*/5 acetate buffer (pH 5.2) and a few drops of toluene were introduced and mixed well. The flask was placed in the thermostat of 38°C. When the solution attained the temperature of thermostat, it was then mi xed with 12 cc. of *M*/15 sodium mentholglucuronate. With the interval of one hour after the mixture, 6 cc. was pipetted out and its free glucuronic acid content was determined in a usual manner. The results are summarised in table VI.

Table VI.

$a = 12.936$

End concentration	Time of Incubation t (h)	Gl. acid set free. x (mgm)	Gl. acid conjugated with menthol $a-x$ (mgm)	$k=\frac{1}{t}\log\frac{a}{a-x}$	$k=\frac{1}{t}\frac{a}{a(a-x)}$	$k=\frac{x}{\sqrt{t}}$
M/90	1	0.380	12.556	0.0129	0.0024	0.380
	2	0.686	12.250	0.0118	0.0022	0.485
	3	1.051	11.885	0.0123	0.0023	0.606
	4	1.372	11.564	0.0122	0.0023	0.686
	5	1.770	11.166	0.0127	0.0025	0.791
	6	1.992	10.944	0.0123	0.0023	0.813
	7	2.260	10.676	0.0119	0.0023	0.850
	8	2.571	10.364	0.0120	0.0024	0.808
	6	2.810	10.136	0.0118	0.0024	0.936
	10	3.108	9.828	0.0119	0.0024	0.982

Incubation at 38°C.

It seems true that the reaction in this enzymatic process is monomolecular although as bimolecular it gives a fairly good constant. This suggests that the reaction velocity is regulated by the rate of the formation of the compound of the enzyme with menthol-

glucuronate as well as by the rate of decomposition of this compounds.

Temperature coefficient of this enzyme reaction was also determined. The results are summarised in table VII.

TABLE VII.

$M/15$ Na-mentholglucuronate	1.0 cc.
$M/5$ acetate buffer (pH 5.2)	2.0 cc.
enzyme sol.	3.0 cc.
toluene	1 drop

$a = 12.936$ $t = 8$ h.

Temperature C.	Gl. acid set free. x(mgm)	Gl. acid conjugated with menthol. $a-x$ (mgm)	$k=\frac{1}{t}\log\frac{a}{a-x}$	$\frac{kt+10}{kt}$
20°	0.089	12.847	0.0004	
25°	0.167	12.769	0.0007	
30°	0.915	12.021	0.0040	3.40
35°	1.525	11.411	0.0068	2.75
40°	2.048	10.888	0.0094	2.71
45°	2.700	10.236	0.0127	2.45
50°	3.226	9.710	0.0156	
			mean value	2.66

According to Van't Hoff the relation between the velocity constant and temperature can be expressed by the following formula :—

$$\frac{dnk}{dT} = \frac{q}{RT^2}$$

where q represents the heat of activation. By integration one can get

$$\log\frac{k_1}{k_2} = \frac{q}{4.573}\left[\frac{1}{T_2} - \frac{1}{T_2}\right]$$

If we take $k_1/k_2 = 2.66$ which is the average value obtained in the range of optimal temperature then the heat of activation is 36340 cal.

III. Effect of ultraviolet ray upon β-glucuronosidase.

The purified enzyme solution was irradiated by the ultraviolet ray (Hanau) at the distance of 30 cm. for 10–50 minutes and its activity was determined. The results are given in table VIII.

Table VIII.

M/15 Na-mentholglucuronate	1.0 cc.
M/5 acetate buffer (pH 5.2)	2.0 cc.
enzyme sol.	3.0 cc.
toluene.	1 drop

Time of irradiation (min)	Gl. acid set free. (mgm)	k'	$\frac{k'}{k}$
10′	1.48	0.00535	0.43
20′	0.78	0.00338	0.27
30′	0.25	0.00105	0.08
40′	0.00	0.00000	0.00
50′	0.00	0.00000	0.00
Before irradiation	2.43	k 0.01255	1.00

Incubation at 38°C for 8 h.

It can be seen that by 10 minutes exposure nearly half of the activity was lost and by 40 minutes completely annihilated.

IV. Effect of neutral salts.

In this case the optimal H-ion concentration was realised by adding diluted acetic acid using methylred as an indicator.

The effect of acetate ion can be neglected, because various salts were added to the end concentration of 1/10 mol. The results are tabulated in table IX.

It comes out from the table that by all of the neutral salts under investigation except Na-citrate the enzyme activity was greatly enhanced.

This effect of various ions can be arranged as follows in the decreasing order.

Table IX.

$M/15$ Na-mentholglucuronate	1.0 cc.
6/10 Mol. salt solution	
6/10 Mol. salt solution (control test is carried out with aq dest.)	1.0 cc.
enzyme sol.	3.0 cc.
acetic acid / aq dest.	1.0 cc.
toluene	1 drop

Cation.

End concentration $M/10$ salt added.	Gl. acid set free. mgm	k'	$\frac{k'}{k}$
$BaCl_2$	2.403	0.01116	2.32
$CaCl_2$	2.257	0.01041	2.16
$MgCl_2$	2.325	0.01076	2.24
NaCl	2.452	0.01141	2.37
Anion.			
KI	2.878	0.01380	2.85
NaCl	2.262	0.01141	2.37
Na_2SO_4	1.606	0.00847	1.76
Na_2HPO_4	1.755	0.00911	1.89
Na-acetate	2.250	0.01055	2.17
Na-citrate	0.532	0.00228	0.47
Control	1.097	0.00481	

Incubation at 38°C $t=2$ h.

Cation: $Na > Ba > Mg > Ca$.

Anion: $I > Cl >$ acetate $> PO_4 > SO_4$.

The explanation of this salt effect can not be given at present, but it seems plausible that the purified enzyme may be of globulin nature and by dialysis its dispersity might be somewhat lessened, and which by the addition of salt is again restored.

V. The effect of organic acid.

In the previous experiment on the salt effect it was found that sodium citrate alone exhibited a retarding action upon β-glucuronosidase which was confirmed by repeated experiments. This peculiar behaviour is worth further investigation. Since this should be ascribed to the configuration of citrate molecule, other various organic acids were tested to collate with this finding, namely, sodium salts of acetic-, propionic-, butyric-, lactic-, oxalic-, malonic-, succinic-, glutaric-, tartronic-, malic-, and tartaric acids were taken into investigations.

The enzyme solution was adjusted to pH 5.2 by diluted acetic acid and was mixed with the solution of the salt above mentioned, the end concentration of which being $M/50$ mol. The results are given in table XI (A–B).

All monocarbonic and bicarbonic acids seem to accelerate the

Table X—A.

$M/15$ Na-mentholglucuronate	1.0 cc.
7/50 Mol. salt solution (control test is carried out with aq dest.)	1.0 cc.
enzyme sol.	3.0 cc.
acetic acid / aq dest.	1.0 cc.
toluene	1 drop

	End concentration $M/50$	Gl. acid set free.		$\frac{k'}{k}$
	salt added	mgm	k'	
monocarbonic acid	acetic acid	2.757	0.01309	1.77
	propionic acid	2.641	0.01240	1.68
	butyric acid	2.512	0.01172	1.59
oxymonocarbonic acid	lactic acid	2.294	0.01060	1.43
	control	k 1.646	0.00739	

Incubation at 38°C $t = 8$ h.

Table X—B.
Incubation at 38°C $t = 8$ h.

	End concentration $M/50$	Gl. acid set free.		
	salt added.	mgm	k'	$\frac{k'}{k}$
di-carbonic acid	oxalic acid	2.693	0 01267	1.92
	malonic acid	2.430	0.01130	1.71
	succinic acid	2.241	0.01033	1.57
	glutaric acid	2.232	0.01028	1.56
oxydi-carbonic acid	tartoronic acid	1.889	0.00857	1.30
	malic acid	0.352	0.00150	0.23
	tartaric acid	1.223	0.00540	0.82
oxytri-carbonic acid	citric acid	1.039	0.00455	0.69
	control.	1.480	k 0.00660	

reaction. Among oxycarbonic acids lactic and tartronic acids also favour the enzyme but in a lesser degree than the above mentioned.

To the contrary tartaric-, citric-, and malic acids showed an inhibiting effect in the increasing order. The inhibition due to malic acid is especially pronounced, the activity of enzyme in its presence being nearly one fifth of the control.

If we compare the chemical structure of glucuronate with that of malic acid we find that β-oxycarboxyl radical (–CHOH.CH. COOH) is common to both molecules.

It may be suggested, therefore, that glucuronate is anchored on the surface of enzyme through this radical, but in the presence of malic acid it is displaced and escapes from the splitting.

A somewhat less effect of retardation of tartaric acids than that of malic may be ascribed to the introduction of –OH group in the α-position to the carboxyl in the named radical and that of citric to the $-CH_2$ COOH groups in α-position.

At any rate it may be said that the essential feature of the

retardation in these compounds may be sought to the radical of β-oxycarbonic acid. The α-oxycarbonic acid has no such an effect can be aknowledged by the behaviour of lactic and tartoronic acids.

Summary.

The kinetics of the action of purified β-glucuronosidase upon mentholglucuronate were studied. The results are summarised as follows:—

1. The optimal pH of this enzyme action lies at 5.0–5.2, under the buffering with acetate mixture.

2. The reaction velocity of this enzyme action proceeds monomolecular.

3. The temperature coefficient shows the average value of 2.66 in the range of 30°–45°C.

Then the heat of activation is 36340 cal.

4. Irradiation of ultraviolet ray produces a strong inhibiting effect.

5. The addition of neutral salt promotes the enzyme action in a remarkable manner.

6. The sodium salts of oxydicarbonic acids such as malic-, tartaric-, and citric acids exhibit a powerful inhibiting effect, while those of lactic-, acetic-, propionic-, and butyric acids rather promote. The plausible explanation for this phenomenon was suggested.

Thanks are due to Prof. K. Kodama and Prof. H. Masamune for their criticisms and encouragement.

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GLYOXALASE AND ITS CO-ENZYME.

III. The Mechanism of the Action of Glutathione as the Co-Enzyme of Glyoxalase.

By

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It was pointed out in a previous paper (Nagaya, Yamazoye and Nakamura (1936)), that glutathione combined with methylglyoxal at an equimolecular ratio in the initial stage of the glyoxalase action, and that glutathione was subsequently liberated almost quantitatively with a concomitant conversion of methylglyoxal into lactic acid. In the meantime, Girsavicius and Heyfetz (1935 ii) gave a brief preliminary report on the similar behavior of glutathione during the glyoxalase action. The present communication deals with the detailed account of the mechanism of the coenzyme-action of glutathione.

I. The Initial Decrease and Subsequent Recovery of Glutathione During the Glyoxalase Action.

On bringing an excess of methylglyoxal into contact with glutathione in the presence of glyoxalase, glutathione showed instantaneously a very rapid fall to a minimum value, and kept this value until a large part of methylglyoxal was converted into lactic acid. When the remaining quantity of methylglyoxal approached the value which was nearly equivalent to glutathione used, glutathione tended distinctly to recover, and reached almost the original value at the moment of the complete conversion of methylglyoxal into lactic acid. By plotting the points which showed the hourly change of the quantity of glutathione during the reaction, a graceful *U*-curve was described. On adding methylglyoxal after the

complete recovery of glutathione, a second curve of a similar form to the first one was obtained. A typical curve is represented in Fig. 1. The glutathione-curves drawn by the methods of Mason (1930), Kühnau (1931 i), Woodward and Fry (1932), Benedict and Gottschall (1933) and Fujita and Iwatake (1935) fell practically in the same family (Table I). The

Fig. 1. Quantitative Changes of Glutathione, Methylglyoxal and Lactic Acid during Glyoxalase Action.

I. 40.0 mg. of glutathione, 54.6 mg. of methylglyoxal, 4 cc. of aqueous extract of rabbit liver (1:4), and 120 cc. of boric acid-borax buffer solution of pH 7.0 were diluted to 200 cc. with water. Temperature was 37°C.

II. After 20 minutes' incubation, 27.3 mg. of methylglyoxal was added anew to 100 cc. of the reacting solution.

Glutathione was determined by the Kühnau method; methylglyoxal by our colorimetric method; lactic acid by the friedemann, Cotonio and Shaffer method after treating the solution with animal charcoal.

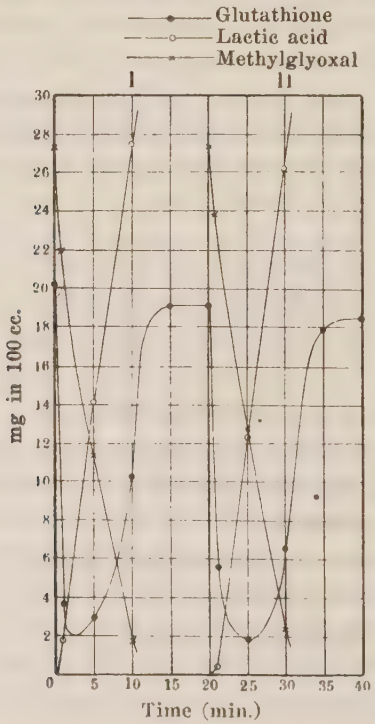

Table I.

Change of Glutathione Quantity Determined by Various Methods during Glyoxalase Action.

In 100 cc.:

28.8 mg. of methylglyoxal, 20.0 mg. of glutathione, 2 cc. of liver extract of rabbit and 60 cc. of boric acid-borax buffer solution of pH 7.0. 37°C.

Methods	Glutathione present (mg. in 100 cc.)						
	1 min.	5 min.	10 min.	12.5 min.	15 min.	17.5 min.	20 min.
Kühnau	2.73	2.98	5.95	11.16	16.62	18.72	19.22
Woodward	2.27	2.80	5.90	—	16.50	—	19.27
Benedict *1	—	—	—	10.80	—	18.90	—
Mason *2	—	—	—	—	15.80	—	19.20
Fujita *3	—	—	—	11.50	—	—	19.50
Methylglyoxal	—	13.24	4.25	—	1.22	—	0

*1 When relatively large amounts of methylglyoxal still remained, they interfered with the determination of glutathione by developing blue colour with the reagents.

*2 Values of glutathione were lower in the presence of larger amounts of methylglyoxal.

*3 The colour became tinged with orange in the presence of relatively large amounts of methylglyoxal. The compound between glutathione and methylglyoxal was slightly decomposed by the alkalinity of the reagents.

Kühnau method was the most suitable for the purpose of demonstrating the enzymic combination of the substances, since the results obtained by it showed most accurately the combination catalyzed by glyoxalase. The inadequacy of other methods will be pointed out later. The minimum value of the glutathione-curve was lower, and the angle with which the decreased glutathione showed the tendency of recovery was sharper when larger quantities of enzyme were employed. The results concerning these points are summarized in Table II. The diminution of glutathione was never reduced to zero under any conditions; there always remained several percent of glutathione in the free state. The recovery of glutathione was not strictly quantitative but never below 90%. The decrease of

Table II.

Reaction between Glutathione and Methylglyoxal with Varying Quantities of Enzyme and Methylglyoxal.

I. In 100 cc.:—
10.0 mg. of glutathione, 4 cc. of glyoxalase solution, 60 cc. of pH 7.0 buffer solution, and a) 10.5 mg. b) 30.0 mg., c) 45.0 mg. of methylglyoxal. 37°C.

II. In 100 cc.:—
10.0 mg. of glutathione, 30.0 mg. of methylglyoxal, 60 cc. of buffer solution, and a) 2 cc., b) 1 cc., c) 0.5 cc. of glyoxalase solution. 37°C.

Glutathione was determined by the Kühnau method.

Experiments		Subtsances determined	Substances persent (mg in 100 cc.)								
			1 min.	3 min.	5 min.	10 min.	20 min.	25 min.	28 min.	50 min.	80 min.
I	a	MG *1	10.60	7.28	3.62	0					
		GSH *2	1.24	1.49	2.60	9.80					
	b	MG	26.70	23.41	19.14	8.38	0				
		GSH	0.62	0.37	0.37	0.99	9.30				
	c	MG	43.17	39.79	35.60	23.41	4.75		0		
		GSH	1.24	0.74	0.25	0.25	1.90		9.18		
II	a	MG	26.12	24.15	21.74	16.10	8.13	4.55			
		GSH	0.74	0.62	0.50	0.87	1.49	6.20			
	b	MG	26.46	26.01	21.10				9.30	0	
		GSH	2.60	1.74	1.92				2.23	9.05	
	c	MG	27.61	26.93	25.36			16.80			0
		GSH	5.95	2.23	2.11			1.81			6.94

*1 MG = methylglyoxal; *2 GSH = glutathione.

glutathione did not mean the change of the reduced form into the oxidized compound. Glutathione had no bearing upon glyoxalase in its disulfide form. Barrenscheen's hypothesis (1932) that oxidized glutathione might participate in the reaction involving the conversion of methylglyoxal into lactic acid was disproved. That the recovered glutathione was actually the glutathione itself was proved by the evidence that the recovered glutathione acted again as the co-enzyme on the second addition of methylglyoxal (Fig. 1). Lohmann's finding (1931) that the quantity of glutathione showed inconstant values during the action of glyoxalase, is explainable by

union and disunion between glutathione and methylglyoxal.

All the facts mentioned above seem to indicate the catalytic action of glyoxalase on the combination of glutathione with methylglyoxal. Therefore it is expected that at a very initial stage of the reaction the decrease of methylglyoxal might mean mainly its combination with glutathione, and no lactic acid would yet be produced; the rate of the conversion of methylglyoxal into lactic acid should be quite low. However, it was observed that the determination of lactic acid by the Friedemann, Cotonio and Shaffer method (1927) after removing methylglyoxal with 2.4-dinitrophenylhydrazine, showed high rates of the conversion even at this early stage of the reaction. The reason for this evidence which was inconsistent with the theoretical assumption was traced to the fact that the compound between methylglyoxal and glutathione was labile enough to form lactic acid when boiled in acid medium under the conditions of the lactic acid determination (see Chapter III). The compound was found to be adsorbed by animal charcoal quantitatively, and easily removed from the reaction system. The loss of lactic acid due to adsorption with charcoal was negligible. Thus, when lactic acid was determined after treating the reacting fluid with animal charcoal, the rate of its production from methylglyoxal was very low during the initial stages of the reaction. However, the rate showed subsequently a gradual increase with the progress of the reaction and it finally reached the theoretical values. The values of lactic acid before and after treating with charcoal are listed side by side in Table III. Thus the quantities of methylglyoxal which have disappeared correspond to the sum of the amounts of the substance which are actually converted into lactic acid and those which are existing in the combined state with glutathione. Then the amounts of methylglyoxal which have decreased as the result of its conversion into lactic acid can be easily calculated. The true rates of the conversion are only reckoned accurately by comparing the quantities of lactic acid really formed with the amounts of methylglyoxal which are computed in the way mentioned above. The table indicates that the true rates experimentally determined are from 93 to 95%. Con-

Table III.

Decrease of Glutathione and Methyglyoxal at Initial Stage of Glyoxalase Action.

In 100 cc.:—
40.0 mg. of glutathione, 25.42 mg. of methyglyoxal, 2 cc. of glyoxalase solution and 60 cc. of pH 7.0 buffer. 37°C.

Glutathione was determined by the Kühnau method.

		Mg. in 100 cc.			
		1 min.	3 min.	6 min.	15 min.
Decrease of glutathione		25.86	34.83	33.34	3.09
Decrease of methylglyoxal		7.52	14.06	20.74	25.42
Production of lactic acid	Before treating with animal charcoal	8.83	15.61	23.17	28.83
	After treating with charcoal	1.70	6.92	15.07	28.57
Rate of conversion of methylglyoxal into lactic acid	After treating with charcoal	15.2%	37.9%	57.6%	89.8%
	True rate*	93.7%	94.2%	93.4%	92.5%

* Ratio between the quantities of lactic acid experimentally determined and those theoretically calculated. The theoretical values meant the quantities of lactic acid which were expected to be formed from the amounts of methylglyoxal corresponding to the difference between the total quantities of the decreased methylglyoxal and those which remained in the state of combination with glutathione.

sidering the accuracy of the method of lactic acid determination (95%), the conversion of methylglyoxal into lactic acid can be regarded as taking place quantitatively. The combination between glutathione and methylglyoxal took place at approximately an equimolecular ratio (1:4.26), when the ratio was reckoned from the amounts of methylglyoxal and glutathione which decreased during the initial stages of the reaction (Table IV).

II. The Combination of Methylglyoxal with Glutathione in Presence and Absence of Glyoxalase.

The direct chemical reaction between the carbonyl group of methylglyoxal and the sulfhydryl group of glutathione has been

Table IV.

Combination of Glutathione with Methylglyoxal at Initial Stage of Glyoxalase Action.

In 100 cc.:—

20.82 mg. of glutathione, 14.78 mg. of methylglyoxal, 60 cc. of pH 7 buffer solution, and 1) 1 cc., 2) 0.8 cc., 3) 0.6 cc., 4) 0.4 cc., of glyoxalase solution. 20°C.

Glutathione was determined by the Kühnau method.

Molecular ratio of glutathione to methylglyoxal = 4.26:

No. of experiments		Time (min.)		
		0.5	1	1.5
1	(a) Decrease of GSH *1 (mg. in 100 cc.)	2.57	5.01	7.63
	(b) Decrease of MG *2 (mg. in 100 cc.)	0.61	1.20	1.74
	Ratio (a) : (b)	4.21	4.17	4.38
2	Decrease of GSH	1.92	4.16	6.25
	Decrease of MG	0.44	0.94	1.46
	Ratio	4.38	4.42	4.28
3	Decrease of GSH	1.60	3.33	4.88
	Decrease of MG	0.35	0.78	1.18
	Ratio	4.57	4.26	4.13
4	Decrease of GSH	1.20	2.15	2.77
	Decrease of MG	0.28	0.52	0.66
	Ratio	4.28	4.13	4.19

*1 GSH = glutathione. *2 MG = methylglyoxal.

repeatedly observed by many investigators. Kühnau (1931), Jowett and Quastel (1933), Platt and Schroeder (1934), and Girsavicius and Heyfetz (1935 i) demonstrated the reaction by the decrease of the iodine-consuming power of glutathione in the presence of methylglyoxal. Lohmann (1932) induced the occurence of the reaction in alkaline medium from the fact that in the presence of glutathione the formation of 2.4-dinitrophenyl-bis-hydrazone of methylglyoxal took place more slowly than in acid medium. It has been generally recognized that the reaction was

more remarkable at lower hydrogen-ion concentratinos. The decrease of the iodine-combining power of glutathione in the presence of methylglyoxal led Jowett and Quastel to the hypothesis that glyoxalase might catalize the combination between these substances and the subsequent production of lactic acid and free glutathione from this compound. The hypothesis was later supported by Platt and Schroeder(1934), and Girsavicius and Heyfetz(1934 i). Special mention is made here that the basis of the hypothesis was strictly confined to one fact that glutathione reacted with methylglyoxal in vitro; the development of this hypothesis, in the biological field, was not accompanied at all by experimental justification with the use of glyoxalase. Though the hypothesis was fortunately justified by the present writer, and Girsavicius and Heyfets (1935 ii), it was rather speculative in view of the fact that this sort of a reaction is by no means specific for methylglyoxal and glutathione. Baumann (1885), Bongartz (1886), Posner (1903), and Fromm and Erfurt (1909) assumed the possible formation of the addition-products between aldehydes and mercaptans. The present writer found that the iodine-combining power of the sulfhydryl substances, such as cysteine and thioglycolic acid, also decreased in the presence of methylglyoxal, in spite of the fact that these sulfhydryl compounds never acted as the coenzyme of glyoxalase. Moreover, Kühnau (1931) observed the reaction between glutathione and acetaldehyd in an acid medium. There is another strong basis against the assumed analogy of English investigators. As will be stated below, the chemical compound between glutathione and methylglyoxal differed decidedly from the compound, the formation of which was catalyzed by glyoxalase, in many points. For the sake of convenience the former compound will be named the chemical compound and the latter the biological compound.

The chemical and biological compounds were isolated in a state of powder in the following way.

Preparation of the chemical compound. 0.20 gm. of glutathione was dissolved in 10 cc. of 0.85 M methylglyoxal (0.612 gm.) and the mixture was put aside at its own acidity (pH 2). Tem-

perature was 30°C. After 3 hours the decrease of glutathione was 98%. The fluid was rapidly dried up by vacuum destillation. The powder formed was dissolved in small quantity of water, and the compound was precipitated with 100 cc. of absolute alcohol and 50 cc. of ether. The precipitate was repeatedly washed with ether until methylglyoxal was completely removed. The compound did not give the characteristic smell of glutathione. The yield was 0.2 gm. The compound was quite labile and was easily decomposed into its components when it was dissolved in water. Owing to this property the compound could not be put through a process of complete purification. The purity was round 90%.

Preparation of biological compound. 1.0 gm. of glutathione and 1.5 gm. of methylglyoxal were dissolved in 800 cc. of water of 37°C. The mixture was neutralized with baryta, and added to 100 cc. of glyoxalase solution which was prepared by extracting 2 gm. of the acetone-dried powder of rabbit liver with 150 cc. of water for 30 minutes at room temperature. The acidity of the fluid was kept at pH 7 by the continuous addition of baryta. After incubating at 37°C for 20 minutes 95% glutathione disappeared. At this stage the reaction was stopped by the addition of 4.7 cc. of 5*N* H_2SO_4 which was approximately correspondent to the baryta added. Barium sulphate was filtered off, the filtrate was concentrated to 30 cc. at 45°C. One gm. of trichloroacetic acid was added to the concentrated fluid, and the protein precipitate was centrifuged off. The supernatant fluid was concentrated further to a few cc., and a small quantity of the precipitate formed was centrifuged off. The clear fluid was freed from trichloroacetic acid by repeated shaking with ether, and dropped into 100 cc. of absolute alcohol. The precipitate formed was washed with ether untill the remaining methylglyoxal was completely removed, and dried. The yield was 0.8 gm. The powder was dissolved in a small quantity of water, and then precipitated with alcohol and ether. This treatment was repeated 7 times, and 0.3 gm. of white powder was obtained. Owing to the obscurity of the mode of the enzymic combination between glutathione and methylglyoxal, the purity was undetermined. The best sample so far obtained was still contaminated with 2.5%

glutathione. The nitrogen content was 10.7%. Upon heating the sample, it disrupted into its components approximately at 160°C.

III. The Difference in Properties of Chemical and Biological Compounds.

a) Stability of the chemical and biological compounds.

One of the most striking differences between chemical and biological compounds was their stability in neutral and alkaline solution. It is clearly demonstrated in Table V that the decomposition of the biological compound, which was indicated by the liberation of glutathione, took place quite slowly at neutral reaction with the simultaneous formation of lactic acid.

Table V.

Stability of Chemical and Biological Compounds in Aqueous Solution.

10 mg. of chemical and biological compounds were dissolved in 20 cc. of pH 7 buffer solution, and diluted to 100 cc. with water. 20°C. Glutathione was estimated by the direct iodimetry in the following way. 10 cc. of the solutio nto be tested were added with 8 cc. of 4% sulfosalicylic acid and 1 cc. of 25% KI, and titrated with $N/500$ KIO_3. Both compounds contained 2.5 mg.% free glutathione as impurity.

	Time (min.)	Mg. of substances produced from 100 mg. of compounds		
		Glutathione	Lactic acid	Methylglyoxal
Biological compound	1	0	0	0
	5	3.7	+	0
	10	7.4	2.3	0
Chemical compound	1	65.7	0	16.27
	5	65.7	0	16.27
	10	65.7	0	16.27

The biological compound was sensitive to alkalinity. Table VI shows that the compound was stable at pH 5, while it liberated glutathione at greater velocity with the increase of alkalinity and temperature. The liberated glutathione had the tendency to decrease after reaching the maximum point. This was due to the

TABLE VI.

Influence of Hydrogen-Ion Concentration and Temperature upon Liberation of Glutathione from Biological Compound.

In 100 cc.:—

40 mg. of the compound, 3 cc. of buffer solution.

Glutathione was determined by the Kühnau method.

pH	Temperature (C)	Mg. of glutathione liberated (in 100 cc.)			
		1 min.	5 min.	10 min.	20 min.
5.0	20°	0	0	0	0
7.0	20°	0	3.66	4.96	—
	37°	0.72	8.41	13.25	15.85
9.0	16°	6.67	16.10	17.84	16.35
	37°	10.18	18.08	15.36	—

susceptibility of the glutathione once formed toward alkalinity. The lactic acid formed from the compound in alkaline medium was identified as Zn-salt.

The biological compound was quite stable in strong acid medium. However, it decomposed and produced lactic acid at high temperature. As listed above in Table III, lactic acid was formed under the conditions of the Friedemann, Cotonio and Shaffer method of the lactic acid determination (at approximately 1 *N* H_2SO_4-acidity and 100°C). The quantity of lactic acid produced increased as the time of heating was prolonged previous to the addition of permanganate. Heating for 30 minutes was not long enough to induce the quantitative production of lactic acid; 10.0 mg. of the compound formed 1.88 mg. of lactic acid by this treatment, while 2.05 mg. were produced after complete decomposition of the compound by keeping it at pH 9 for 20 minutes at room temperature. The rate of the production of lactic acid from the compound was greater with the employment of smaller quantities of the compound.

The chemical compound was not stable even in acid medium. 10 mg. of the compound dissolved in 100 cc. of 0.7% HCl solution, yielded 0.25, 0.31, 0.37, 0.62, 1.24 and 2.23 mg. of glutathione after

standing at room temperature for 1, 5, 10, 30, 60 and 120 minutes respectively. The chemical compound was decomposed into glutathione and methylglyoxal with greater velocity as the hydrogen-ion concentration of the medium decreased. The decomposition took place momentarily at neutral reaction (Table V). Lactic acid was never produced under any conditions, unless the decomposition was carried out by glyoxalase. The instability of the addition-products between aldehydes and mercaptans was pointed out by Baumann and others.

All the facts just mentioned indicate the unmistakable difference in the properties of biological and chemical compounds. The biological compound was far more stable in comparison with the other compound, and it produces, when it is decomposed, lactic acid and free glutathione; but methylglyoxal is never liberated. On the other hand the chemical compound is extremely labile in the solution. The decomposition means always the liberation of its components, glutathione and methylglyoxal; lactic acid is not formed.

b) Behavior of chemical and biological compounds towards iodimetric methods for the determination of glutathione.

The chemical combination between glutathione and methylglyoxal was demonstrable by the direct titration with iodine solution. This combination was also detectable, though to a less extent than by the direct titration method, by the Kühnau method of re-titrating excessive iodine with thiosulphate, when a large quantity of methylglyoxal was present as in the experiments of Kühnau(1931 ii). However, Yamazoye(1933) pointed out that the combination was scarcely noticed by means of the Kühnau method when relatively a small quantity of methylglyoxal was used. This was due to the disruption of the linkage between two substances in the presence of excessive iodine, but not to the dilution effect as suggested by Girsavicius and Heyfetz (1935 i). The effect of excessive iodine was more marked with the prolongation of the contract of iodine with the compound.

Contrary to the chemical compound, the biological compound was stable in excessive iodine. The Kühnau method and direct iodimetric methods gave similar results. The difference between the two compounds with respect to their behavior towards idodimetric methods is demonstrated in Table VII. It is shown in the table that both compounds contained 2.5 percent of free glutathione as impurity.

TABLE VII.

Direct and Indirect Iodimetry of Chemical and Biological Compounds.

Direct titration:

1 cc. of 100 mg.% solution of the compounds was mixed with 18 cc. of 4% trichloroacetic acid solution, 1 cc. of 25% KI-solution. The mixture was titrated with $n/500 KIO_3$-solution.

Indirect titration (modified Kühnau's method):

1 cc. of the solution was mixed with 18 cc. of 4% trichloroacetic acid solution, 1 cc. of 25% KI-solution and 2 cc. of $n/200$ KIO_3 and titrated with $n/500$ thiosulphate solution.

	Time of titration	Mg. of glutathione in 100 mg. of compounds	
		Direct titration	Indirect titration
Biological compound	Immediately	2.5	2.5
	After 2 minutes	2.5	2.5
Chemical compound	Immediately	2.5	36.0
	After 2 minutes	2.5	71.9

It is easily understood from the facts mentioned above why the direct chemical iodimetric methods do not give accurate results of the enzymic combination. These methods include not only the enzymic combination, but also the chemical one which takes place between glutathione and methylglyoxal remaining in the reacting medium. On the other hand the Kühnau method excludes the chemical combination, responding to the enzymic reaction only.

c) *Action of glyoxalase on biological and chemical compounds.*

When the biological compound was brought to contact with

the coenzyme-free glyoxalase, it was rapidly decomposed into glutathione and lactic acid. The products were formed at an equimolecular ratio (Table VIII). Methylglyoxal was not liberated. Thus it is clear that glyoxalase catalyzes the reaction which involves the decomposition of the biological compound into lactic acid and glutathione.

Table VIII.

Decomposition of Chemical and Biological Compounds by Glyoxalase.

In 100 cc.:—

50.0 mg. of compounds, 4 cc. of coenzyme-free glyoxalase solution prepared from the acetone-dried rabbit liver. 60 cc. of pH 7 buffer solution. 37°C.

Control experiment was run without glyoxalase.

Glutathione was determined by the Kühnau method.

Lactic acid was measured after the removal of the remaining compound with animal charcoal.

	Substances determined	Substances produced (mg. in 100 cc.)					
		0 min.	1 min.	2 min.	5 min.	10 min.	15 min.
Biological compound	Glutathione	1.25	—	15.87	30.26	37.20	36.70
	Lactic acid	0	—	4.01	8.11	10.14	10.30
	Methylglyoxal	0	—	0	0	0	0
Chemical compound	Glutathione	35.95	18.86	14.40	23.08	33.74	33.24
	Lactic acid	0	0.78	1.89	5.17	10.14	10.11
	Methylglyoxal	8.15	3.17	1.16	+	0	0

As the chemical compound was disrupted into its components in aqueous solution, the circumstance under which glyoxalase acted was just the same as in the case where the enzyme acted on methylglyoxal in the presence of glutathione. The glutathione which was liberated from the compound gave the typical *U*-curve, and methylglyoxal disappeared with the simultaneous production of lactic acid.

IV. Discussion.

Evidence has been presented in the foregoing chapters that the biological compound differs from the chemical one in several

ways. It emphasizes a difference between test tube chemistry and biochemistry.

In the case of the chemical compound, glutathione and methylglyoxal might react, as Baumann and others suggested in accordance with the general equation which involves the formation of the addition-products between aldehydes and mercaptans:

$$R.CHO + R'SH = R.CH(OH).SH'$$

Jowett and Quastel expressed presumably the reaction in the following manner:

$$CH_3.CO.CHO + GSH \rightleftarrows CH_3.CO.C\begin{matrix}OH\\GS\\H\end{matrix}$$

The equilibrium between the chemical compound and its components is easily influenced by dilution.

Although glutathione and methylglyoxal react at an equimolecular ratio also in the biological compound, there we are not dealing with the same kind of reaction as in the other case. The enzymic combination of glutathione with methylglyoxal seems to introduce into the molecule of the latter substance some change which causes methylglyoxal to be converted into lactic acid when the compound is decomposed in any way what-so-ever. There may be another possibility, that glyoxalase introduces the change before methylglyoxal combines with glutathione. The true feature of the biological reaction has not yet been elucidated. The biological compound contained no free carbonyl group in its molecule; the 2.4-dinitrophenylhydrazine reaction and the fuchsinsulphurous acid reaction were negative.

Summary.

1) When methylglyoxal is converted into lactic acid by the action of glyoxalase, the reaction begins with the combination between methylglyoxal and glutathione at an equimolecular ratio. Glyoxalase is absolutely necessary for this reaction. The compound between methylglyoxal and glutathione has been isolated in a state of powder. Though it is stable in acid solution, it is decomposed

into lactic acid and glutathione gradually in neutral solution, and rapidly at alkaline reaction. The decomposition is catalyzed by glyoxalase. The compound is stable against iodine.

2) Methylglyoxal can combine directly with glutathione in the absence of glyoxalase, and this chemical compound has been also isolated. The compound is very labile. It is hydrolyzed simply into glutathione and methylglyoxal in aqueous solution. The velocity of the hydrolysis is relatively slow in acid medium while the compound is hydrolyzed instantaneously in neutral or alkaline medium. The decomposition of the compound is never accompanied by the formation of lactic acid. The chemical compound is decomposed by iodine.

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